

JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application: March 18, 2003

Application Number: Patent Application No.2003-074573

Applicant(s): KEIO UNIVERSITY

Commissioner, Yasuo Imai (Sealed)
Patent Office

Name of Document	Patent Application
Case Number	000000448
Filing Date	March 18, 2003
Addressee	Commissioner of Patent Office
International Classification	C12N 5/06
Inventor	
Address or Residence	35, shinano-cho, shinjuku-ku, Tokyo % School of Medicine, Keio University
Name	Masataka KUWANA
Inventor	
Address or Residence	35, shinano-cho, shinjuku-ku, Tokyo % School of Medicine, Keio University
Name	Hiroaki KODAMA
Applicant for Patent	
Code Number	899000079
Name or Corporation Name	KEIO UNIVERSITY
Agent	
Code Number	100107984
Patent Attorney	
Name or Corporation Name	Masanori HIROTA
Appointed Agent	
Code Number	100102255
Patent Attorney	
Name or Corporation Name	Seiji OZAWA
Appointed Agent	
Code Number	100118957
Patent Attorney	
Name or Corporation Name	Haruko OKA

Indication of Fee

Deposit Account Number 044347

Amount of Payment 21,000 yen

List of Filed Documents

Name of Document Scope of Claims 1

Name of Document Specification 1

Name of Document Drawing 1

Name of Document Abstract 1

Confirmation of Proof Proof Necessary

Name of Document SPECIFICATION

Title of the Invention MONOCYTE-ORIGIN MULTIPOTENT CELL MOMC

Scope of Claims

Claim 1 A monocyte-derived multipotent cell, derived from a monocyte, which expresses CD14 and CD34.

Claim 2 A monocyte-derived multipotent cell, derived from a monocyte, which expresses CD14, CD34, CD45 and type I collagen.

Claim 3 The monocyte-derived multipotent cell according to claim 1 or 2, that can differentiate into mesenchymal cells by a culture under a condition inducing differentiation into mesenchymal tissues.

Claim 4 The monocyte-derived multipotent cell according to claim 3, wherein the mesenchymal cells are osteoblasts, skeletal myoblasts, chondrocytes or adipocytes.

Claim 5 The monocyte-derived multipotent cell according to claim 1 or 2, that can differentiate into myocardial cells by a culture under a condition inducing differentiation into cardiac muscle such as a coculture with cultured myocardial cells.

Claim 6 The monocyte-derived multipotent cell according to claim 1 or 2, that can differentiate into nerve by a culture under a condition inducing differentiation into nerve, such as a coculture with cultured nerve.

Claim 7 The monocyte-derived multipotent cell according to claim 1 or 2, that can differentiate into endothelial cells by a culture under a condition inducing differentiation into endothelium, such as a culture under a condition maintaining endothelial cells.

Claim 8 The monocyte-derived multipotent cell according to claim 1 or 2, that can differentiate into mesodermal cells.

Claim 9 A method for preparing a monocyte-derived multipotent

cell, comprising culturing peripheral blood mononuclear cells (PBMCs) in vitro on fibronectin, and collecting fibroblast-like cells expressing CD14 and CD34.

Claim 10 The method for preparing a monocyte-derived multipotent cell according to claim 9, comprising culturing in vitro on fibronectin for 5 to 14 days.

Claim 11 A mesenchymal progenitor, a mesenchymal cell or a mesenchymal tissue induced by culturing the monocyte-derived multipotent cell according to any one of claims 1 to 8, under a condition inducing differentiation into mesenchymal tissues.

Claim 12 The mesenchymal progenitor, the mesenchymal cell or the mesenchymal tissue according to claim 11, wherein the mesenchymal cells are osteoblasts, skeletal myoblasts, chondrocytes or adipocyte.

Claim 13 A myocardial progenitor, a myocardial cell or a myocardial tissue induced by culturing the monocyte-derived multipotent cell according to any one of claims 1 to 8, under a condition inducing differentiation into cardiac muscle such as a coculture with cultured myocardial cells.

Claim 14 A neural progenitor, a neuron or a nerve tissue induced by culturing the monocyte-derived multipotent cell according to any one of claims 1 to 8, under a condition inducing differentiation into nerve, such as a coculture with cultured neuron.

Claim 15 An endothelial progenitor, an endothelial cell or an endothelial tissue induced by culturing the monocyte-derived multipotent cell according to any one of claims 1 to 8, under a condition inducing differentiation into endothelium, such as a culture under a condition maintaining endothelial cells.

Claim 16 A mesodermal progenitor, a mesodermal cell or a mesodermal tissue induced to differentiate from the monocyte-derived

multipotent cell according to any one of claims 1 to 8.

Claim 17 A therapeutic agent comprising as active ingredient the monocyte-derived multipotent cell according to any one of claims 1 to 8 and/or mesodermal progenitors, mesodermal cells and/or mesodermal tissues induced to differentiate from the monocyte-derived multipotent cell.

Claim 18 A therapeutic agent comprising as active ingredient the monocyte-derived multipotent cell according to any one of claims 1 to 8 and/or neural progenitors, neurons and/or nerve tissues induced to differentiate from the monocyte-derived multipotent cell.

Claim 19 A treating method comprising administering the monocyte-derived multipotent cell according to any one of claims 1 to 8 and/or mesodermal progenitors, mesodermal cells and/or mesodermal tissues induced to differentiate from the monocyte-derived multipotent cell.

Claim 20 A treating method comprising administering the monocyte-derived multipotent cell according to any one of claims 1 to 8 and/or neural progenitors, neurons and/or nerve tissues induced to differentiate from the monocyte-derived multipotent cell.

Detailed Description of the Invention

0001

Technical Field to Which the Invention Pertains

The present invention relates to a monocyte-derived multipotent cell of CD14⁺ and CD34⁺ that can differentiate into mesoderm cells such as mesenchymal cells, myocardial cells, and endothelial cells or into neurons; a mesodermal cell/tissue such as mesenchymal cell, myocardial cell and endothelial cell, and neuron/nerve tissue induced to differentiate from the

monocyte-derived multipotent cell; a therapeutic agent comprising these as active ingredient; and to a treating method comprising administering these.

0002

Prior Art

Peripheral blood monocytes are derived from bone marrow hematopoietic stem cells and are known to differentiate into several phagocytes, including macrophages, dendritic cells, osteoclasts, microglial cells of central nervous system, and liver Kupffer cells (e.g., see nonpatent documents 1-4). The differentiation of the monocytes into various phagocytes is controlled by signaling of various growth factors. In other words, differentiation into macrophages, dendritic cells, osteoclasts are controlled by signaling of M-CSF, GM-CSF and IL-4, and receptor activating factors of NF ligand (RANKL) or M-CSF, respectively (e.g., see nonpatent documents 2, 3 and 5). Until recently, it was believed that the differentiation potential of monocytes was restricted to phagocytes. However, recent studies have shown that human monocytes can differentiate into endothelial-like cells by culturing in vitro with a combination of angiogenic factors (e.g., see nonpatent documents 6 and 7). In addition, the expression of bone-specific alkaline phosphatase was reported during the monocyte differentiation process in the in vitro granuloma model (e.g., see nonpatent document 8). However, the differentiation potential is not completely clarified and it was not known whether monocytes had differentiation potential into cell types other than phagocytes.

0003

On the other hand, it was revealed that many adult tissues contain populations of stem cells that can self-replicate and

give rise to daughter cells that undergo an irreversible terminal differentiation (e.g., see nonpatent document 9). The best-characterized are hematopoietic stem cells and their progeny, but stem cells are identified in most of the tissues, including mesenchymal, neuron, and hematopoietic cells (e.g., see nonpatent documents 10-12). Mesenchymal stem cells (MSCs) are identified as adherent fibroblast-like cells in the bone marrow with differentiation potential into mesenchymal tissues, including bone, cartilage, fat, muscle, and bone marrow stroma (e.g., see nonpatent document 10). Recently, mesenchymal progenitors having morphologic and phenotypic features and differentiation potentials similar to MSCs have been reported at extremely low frequencies in umbilical cord blood (e.g., see nonpatent document 13), as well as in fetal (e.g., see nonpatent document 14) and adult peripheral blood (e.g., see nonpatent document 15). However, MSCs and circulating MSC-like cells do not express various hematopoietic markers or the stem cell/endothelial marker CD34 (e.g., see nonpatent documents 10 and 13-15).

0004

As described above, various postnatal tissue-specific stem cells and embryonic stem (ES) cells are currently being analyzed as candidate sources for future therapeutic intervention for tissue regeneration (e.g., see nonpatent document 9). It has been reported that bone marrow-derived MSCs engraft in many organs and differentiate along tissue-specific lineages upon its transplantation in animal models (e.g., see nonpatent documents 17 and 18), as well as in human infant suffering osteogenesis imperfecta (e.g., see nonpatent 16). However, MSCs are rare in adult human bone marrow (0.01% to 0.001%), and expansion of MSCs to the number of cells required for regeneration therapy

is technically difficult, expensive, and time-consuming (e.g., see nonpatent document 19). ES cells are multipotent cells derived from germinal cells that can be propagated indefinitely in vitro being still undifferentiated and induced to differentiate to most cell types in vivo (e.g., see nonpatent 20). Although ES cells have been isolated from human, their use in research as well as therapeutic is cumbered by ethical considerations (e.g., see nonpatent document 21).

0005

Several different precursors that can differentiate into endothelial or mesenchymal cell types have been reported in human postnatal peripheral blood, including endothelial cells (e.g., see nonpatent document 22), smooth muscle cells (e.g., see nonpatent document 23), and mesenchymal cells (e.g., see nonpatent document 15). In vitro expansion of endothelial and smooth muscle progenitors requires a combination of several growth factors (e.g., see nonpatent documents 22 and 23). Mesenchymal progenitors can be expanded in a medium supplemented with 20% fetal bovine serum (FBS) without any additional growth factors, but their development in PBMC cultures was reported to be unaffected by eliminating CD14⁺ cells (e.g., see nonpatent document 15). However, these endothelial or mesenchymal cells do not have the phenotypic characteristics to be positive to CD 14, CD45, CD34 and type I collagen.

0006

Nonpatent document 1: Bioessays, 17, 977-986, 1995

Nonpatent document 2: Blood, 98, 2544-5254, 2001

Nonpatent document 3: BMC Immunol., 3, 15, 2002

Nonpatent document 4: Microsc Res Tech., 39, 350-364, 1997

Nonpatent document 5: J Exp Med., 190, 1741-1754, 1999

Nonpatent document 6: Differentiation, 65, 287-300, 2000
Nonpatent document 7: Cardiovascular Res., 49, 671-680, 2001
Nonpatent document 8: Immunobiology, 202, 68-81, 2000
Nonpatent document 9: Science, 287, 1442-1446, 2000
Nonpatent document 10: Science, 284, 143-147, 1999
Nonpatent document 11: Science, 287, 1433-1438, 2000
Nonpatent document 12: J. Hepatol., 29, 676-682, 1998
Nonpatent document 13: Br. J. Haematol., 109, 235-242, 2000
Nonpatent document 14: Blood, 98, 2396-2402, 2001
Nonpatent document 15: Arthritis Res., 2, 477-488, 2000
Nonpatent document 16: Nat. Med., 5, 309-313, 1999
Nonpatent document 17: Nat. Med., 6, 1282-1286, 2000
Nonpatent document 18: Science, 279, 1528-1530, 1998
Nonpatent document 19: Stem Cells, 19, 180-192, 2001
Nonpatent document 20: Trends Biotechnol., 18, 53-57, 2000
Nonpatent document 21: Science, 287, 1397, 2000
Nonpatent document 22: Science, 275, 964-967, 1997
Nonpatent document 23: Circulation, 106, 1199-1204, 2002

0007

An Object to be Attained

The big object remaining in modern medicine is said to overcome deficiency of organs due to disease or external injuries or functional impairment. The only method that can be practiced today for treating such condition is organ transplantation. However, there are still many difficulties for spreading as an actual treating method, due to problems such as brain-death diagnosis or supply from donors. On the other hand, regenerative medicine intending regeneration of organs draws attention with the recent development of stem cells and developmental biology, and is expected as the direction of the medicine to advance in

the 21st century. In animal models, functional recoveries of organs by transplantation of ES cells have been reported, while the application in human is stalled due to rejection or ethical problems of the use of ES cells. Further, as various adult tissue stem cells (mesenchymal, blood vessels, liver etc.) are extremely few in vivo, the isolation thereof is technically difficult, and it is hard at the present time to obtain sufficient amount of cells for transplantation. Therefore, there are many problems to be solved before the regenerative medicine using ES cells or tissue stem cells can be applied to the actual medicine. Particularly, it is essential to supply cells having differentiation potential in a stable manner so that regenerative medicine becomes a reality.

0008

The object of the present invention is to provide a multipotent cell that can differentiate into various cells such as mesenchymal cells including bone, cartilage, skeletal muscle and fat, endothelial cells, myocardial cells and neurons wherein a sufficient amount can be supplied stably with minimum invasion, without problems such as securing donors and rejection in cell transplant, and with less ethical considerations; a mesodermal progenitor/mesodermal cell/mesodermal tissue and a neuron/nerve tissue, such as mesenchymal cells, myocardiac cells, endothelial cells, being induced to differentiate from the multipotential cell; a therapeutic agent comprising these as active ingredient; and a treating method administering the same.

0009

Means to Attain the Object

The present inventors confirmed the expression of fibroblast-like cells when peripheral blood mononuclear cells

(PBMCs) are cultured on fibronectin-coated plastic plates for 7 to 10 days. Being interested by the origin and physiological function of this human cell population exhibiting a fibroblast-like morphology, the present inventors found that these cells are derived from circulating CD14⁺ monocytes, with a unique phenotype of CD14⁺CD45⁺CD34⁺ type I collagen⁺, and having a potential to differentiate into mesenchymal cells including bone, cartilage, smooth muscle and fat, endothelial cells, myocardial cells, neurons, under a particular culture condition. They named this cell the monocyte-derived multipotent cell (MOMC). With the knowledge having revealed for the first time that circulating monocytes are multipotent progenitors having a differentiation potential not only into phagocytes but also into various mesenchymal cells, the present inventors have thus completed the invention.

0010

In other words, the present relation relates to: a monocyte-derived multipotent cell, derived from a monocyte, which expresses CD14 and CD 34 ("1"); a monocyte-derived multipotent cell, derived from a monocyte, which expresses CD14, CD34, CD45 and type I collagen ("2"); the monocyte-derived multipotent cell according to "1" or "2", that can differentiate into mesenchymal cells by a culture under a condition inducing differentiation into mesenchymal tissues ("3"); the monocyte-derived multipotent cell according to "3", wherein the mesenchymal cells are osteoblasts, skeletal myoblasts, chondrocytes or adipocytes ("4"); the monocyte-derived multipotent cell according to "1" or "2", that can differentiate into myocardial cells by a culture under a condition inducing differentiation into cardiac muscle such as a coculture with cultured myocardial cells ("5"); the monocyte-derived multipotent cell according to "1" or "2", that

can differentiate into neuron by a culture under a condition inducing differentiation into nerve, such as a coculture with cultured neuron ("6"); the monocyte-derived multipotent cell according to "1" or "2", that can differentiate into endothelial cells, by a culture under a condition inducing differentiation into endothelium such as a culture under a condition maintaining endothelial cells ("7"); and the monocyte-derived multipotent cell according to "1" or "2", that can differentiate into mesodermal cells ("8").

0011

Furthermore, the present invention relates to a method for preparing a monocyte-derived multipotent cell, comprising culturing peripheral blood mononuclear cells (PBMCs) in vitro on fibronectin, and collecting fibroblast-like cells expressing CD14 and CD34 ("9"); the method for preparing a monocyte-derived multipotent cell according to "9", comprising culturing in vitro on fibronectin for 5 to 14 days ("10"); a mesenchymal progenitor, a mesenchymal cell or a mesenchymal tissue induced by culturing the monocyte-derived multipotent cell according to any one of "1" to "8", under a condition inducing differentiation into mesenchymal tissues ("11"); the mesenchymal progenitor, the mesenchymal cell or the mesenchymal tissue according to "11", wherein the mesenchymal cells are osteoblasts, skeletal myoblasts, chondrocytes or adipocytes ("12"); a myocardial progenitor, a myocardial cell or a myocardial tissue induced by culturing the monocyte-derived multipotent cell according to any one of "1" to "8", under a condition inducing differentiation into cardiac muscle such as a coculture with cultured myocardial cells ("13"); a neural progenitor, a neuron or a nerve tissue induced by culturing the monocyte-derived multipotent cell according to any one of

"1" to "8", under a condition inducing differentiation into nerve, such as a coculture with cultured neuron ("14"); an endothelial progenitor, an endothelial cell or an endothelial tissue induced by culturing the monocyte-derived multipotent cell according to any one of "1" to "8", under a condition inducing differentiation into endothelium, such as a culture under a condition maintaining endothelial cells ("15"); and a mesodermal progenitor, a mesodermal cell or a mesodermal tissue induced to differentiate from the monocyte-derived multipotent cell according to any one of "1" to "8" ("16").

0012

Moreover, the present invention relates to a therapeutic agent comprising as active ingredient the monocyte-derived multipotent cell according to any one of "1" to "8" and/or mesodermal progenitors, mesodermal cells and/or mesodermal tissues induced to differentiate from the monocyte-derived multipotent cell ("17"); a therapeutic agent comprising as active ingredient the monocyte-derived multipotent cell according to any one of "1" to "8" and/or neural progenitors, neurons and/or nerve tissues induced to differentiate from the monocyte-derived multipotent cell ("18"); a treating method comprising administering the monocyte-derived multipotent cell according to any one of "1" to "8" and/or mesodermal progenitors, mesodermal cells and/or mesodermal tissues induced to differentiate from the monocyte-derived multipotent cell ("19"); and a treating method comprising administering the monocyte-derived multipotent cell according to any one of "1" to "8" and/or neural progenitors, neurons and/or nerve tissues induced to differentiate from the monocyte-derived multipotent cell ("20").

0013

Mode for Carrying out the Invention

As for the monocyte-derived multipotent cell (MOMC) of the present invention, there is no specific limitation as long as it is derived from a monocyte, and it is a cell or a cell population expressing CD14, CD34, CD45 and type I collagen, derived from a cell, a cell population or a monocyte expressing CD14 and CD34. The origin of the above monocytes is not particularly limited including mice, rats, dogs, pigs, monkey and human, and human being preferable. As for human monocyte, it can be a monocyte from a donor, while an autologous one is preferable. As for the source of monocytes, monocytes derived from peripheral blood or bone marrow, or monocytes differentiated from hematopoietic stem cells ex vivo can be exemplified. The monocytes herein mentioned are defined to be positive to CD14 or CD11b.

0014

The above-mentioned CD14 and CD45 are known to be a marker of monocyte and cell derived from monocyte, CD34 a marker of endothelial and stem cells, and type I collagen a marker of mesenchymal cells. As for the monocyte-derived multipotent cell of the present invention, those expressing CD105 and Sca-1 as stem cell markers, type III collagen and fibronectin as mesenchymal cell markers, and VE cadherin and Flt-1 as endothelial markers are preferable. The MOMCs are cells distinct from monocytes, macrophages or dendritic cells from their protein expressing pattern mentioned above, and it can be said that MOMCs are a new cell population having combined the characteristics of mesenchymal cells, endothelial cells and stem cells.

0015

The MOMCs of the present invention are derived from circulating CD14⁺ monocytes, as cited in the following. First, MOMCs are

positive for the monocyte lineage markers CD14, CD13, CD11b, CD11c and CD64. Second, serial phenotypic analyses of adherent peripheral blood cells cultured on fibronectin showed increased expression of CD34 on the adherent CD14⁺ cells. Third, the development of MOMCs was almost completely inhibited by depleting the PBMCs of CD14⁺ cells, but not by depleting the PBMCs of cells positive for CD34 or CD105. Finally, studies using labeled-CD14⁺ cells revealed up-regulated expression of CD34 on CD14⁺ monocytes, and that there was no cell proliferating rapidly in PBMC cultures among the CD14⁻ cell population.

0016

As for the MOMCs of the present invention, a cell with multipotency that can differentiate into mesodermal cells under induction condition known to differentiate MSC into mesodermal cells is preferable, particularly a cell with multipotency that can differentiate into mesenchymal cells such as osteoblasts, skeletal myoblasts, chondrocytes, adipocytes, bone marrow stromal cells and smooth muscle cells, by a culture under a condition inducing differentiation into mesenchymal tissues; multipotency that can differentiate into myocardial cells by a culture under a condition inducing differentiation into cardiac muscle such as a coculture with cultured myocardial cells; multipotency that can differentiate into endothelial cells, by a culture under a condition inducing differentiation into endothelium, such as a culture under a condition maintaining endothelial cells; as well as multipotency that can differentiate into neurons that are ectodermal cells, by a culture under a condition inducing differentiation into nerve, such as a coculture with cultured neuron.

0017

Furthermore, the present invention relates to a mesodermal progenitor, a mesodermal cell or a mesodermal tissue induced to differentiate from the MOMCs of the present invention under induction condition known to differentiate MSC to mesodermal cells; for instance a mesenchymal progenitor, a mesenchymal cell or a mesenchymal tissue such as osteoblasts, skeletal myoblasts, chondrocytes and adipocytes, induced by a culture under a condition inducing differentiation of MOMCs into mesenchymal tissues; a myocardial progenitor, a myocardial cell, or a myocardial tissue induced by a culture under a condition inducing differentiation of MOMCs into cardiac muscle such as a coculture with cultured myocardial cells; an endothelial progenitor, an endothelial cell or an endothelial tissue induced by a culture under a condition inducing differentiation of MOMCs into endothelium, such as a culture under a condition maintaining endothelial cells; as well as an ectodermal neural progenitor, a neuron or a nerve tissue induced by a culture under a condition inducing differentiation of MOMCs into nerve, such as a coculture with cultured neuron.

0018

The osteoblasts induced by culturing under a condition inducing differentiation into osteoblasts, are preferably cells with a cylinder-like form, being Alizarin red positive for calcium deposition, alkaline phosphatase staining positive, showing increase of intracellular Ca concentration, and expressing bone sialoprotein II, osteocalcin gene specific to osteoblasts. The chondrocytes induced by a culture under a condition inducing differentiation into chondrocytes are preferably cells slightly large with a cylinder shape, rich of cytoplasm, and expressing type II and X collagens, which are specific to chondrocytes. The skeletal muscle cells induced by culturing under a condition

inducing differentiation into skeletal muscle cells, are preferably cells with a long spindle shape, expressing skeletal muscle-specific actin and myocin. The adipocytes induced by a culture under a condition inducing differentiation into adipocytes are preferably cells with lipid droplets for Oil-red staining positive, wherein PPAR γ and aP2 genes expression is enhanced.

0019

As for the myocardial progenitors or myocardial cells induced by a culture under a condition inducing differentiation into myocardial, cells expressing Nestin, myocardial-specific transcription factors Nkx2.5 and eHAND, and myosin light chain gene, a myocardial cell-structural protein, can be preferably exemplified. As for the neural progenitors or neurons induced by a culture under a condition inducing differentiation into nerve, cells expressing Nestin, and neuron-specific transcription factors NeuroD, Neurogenin 2, and then expressing Hu, and NeuN being nerve markers can be preferably exemplified. As for the endothelial progenitors or endothelial cells induced by a culture under a condition inducing differentiation into endothelium, cells with a polymorphic form with small projections, expressing endothelial-specific marker protein, KDR, vWF and eNOS can be preferably exemplified.

0020

As described in the above, MOMCs have a differentiation potential into mesodermal cells such as mesenchymal cells under induction condition known to mainly differentiate MSC into mesodermal cells, as well as a differentiation potential into neurons of ectodermal system induced by a culture under a condition inducing differentiation of MOMCs into nerve. Moreover, the

differentiation of MOMCs into mesodermal cells such as individual mesenchymal cells follows the steps observed in MSC differentiation, in terms of the timing of lineage-specific transcription factor expression. For example, the expression of C1aloprotein II and osteocalcin follows the expression of Cbfa1/Runx2 (Cell, 108, 17-29, 2002), and further, the expression of MyoD precedes the expression of SkM-actin and myosin (Front Biosci., 5, D750-767, 2000). These findings suggest that differentiation processes into individual mesenchymal lineages are shared by MOMCs and MSCs.

0021

As for the preparation method of MOMCs of the present invention, there is no specific limitation as long as it is a method comprising culturing peripheral blood mononuclear cells (PBMCs) in vitro on fibronectin, for example on plastic plates coated with fibronectin preferably for 5 to 14 days, more preferably for 7 to 10 days, and collecting fibroblast-like cells expressing CD14 and CD34, but it is preferable to culture PBMCs without any additional growth factors. Collagen or laminin can be used instead of the above-mentioned fibronectin, but the differentiation of monocytes into MOMCs requires soluble factors derived from CD14⁺ cells beside fibronectin. The origin of the above PBMCs is not particularly limited including experimental animals such as mice, rats, dogs, pigs and monkeys or human, while human PBMCs can be preferably exemplified. Moreover, human PBMCs can be isolated from human venous blood by common methods. The above-mentioned culturing method is not limited, and a culturing method comprising culturing at 37°C, with 5% CO₂ in a humidified atmosphere, at a density of 10⁴ to 10⁷/ml, for example at 2 × 10⁶/ml, and depleting non-adherent cells and supplementing a

fresh medium every 2 to 4 days, more preferably every 3 days. Thus obtained MOMCs of the present invention can be expanded in culture without losing their original phenotype for up to 5 passages.

0022

The present invention relates to a therapeutic agent comprising as active ingredient the MOMCs of the present invention and/or mesodermal progenitors, mesodermal cells and/or mesodermal tissues induced to differentiate from the MOMCs, for instance, mesenchymal progenitors such as osteoprogenitors, skeletal muscle progenitors, chondroprogenitors, adipoprogenitors, mesenchymal cells such as osteoblasts, skeletal myoblasts, chondrocytes and adipocytes, mesenchymal tissue such as bone, cartilage, muscle and fat, induced by culturing MOMCs under a condition inducing differentiation into mesenchymal tissues; myocardial progenitors, myocardial cells or myocardial tissues induced by culturing MOMCs under a condition inducing differentiation into cardiac muscle, such as a coculture with cultured myocardial cells; endothelial progenitors, endothelial cells, endothelial tissues induced by culturing MOMCs under a condition inducing differentiation into endothelium such as a culture under a condition maintaining endothelial cells. The present invention also relates to a therapeutic agent comprising as active ingredient the MOMCs of the present invention and/or neural progenitors, neurons and/or nerve tissues induced to differentiate from the MOMCs. Further, it relates to a treating method comprising administering the MOMCs of the present invention and/or the mesodermal progenitors, mesodermal cells and/or mesodermal tissues, the neural progenitors, neurons and/or nerve tissues induced to differentiate from the MOMCs, for example administering directly to an impaired or deleted

site or to its proximity or to the peripheral blood. It is preferable to determine appropriately either of MOMCs or MOMCs treated by inducing differentiation are suitable for the therapeutic agent, according to the type of cells or diseases, or administering method. Further, as MOMCs are cells relatively easy to transfer genes, it can be used for tissue reconstitution therapy after introducing a particular gene before cell transplantation to human. For example, when there is some osteogenic impairment in a certain congenital disease, it is possible to transplant after modifying the gene or to prepare it to generate a particular protein (cytokine, growth factor, hormone, etc.).

0023

As described above, as MOMCs have a potential to differentiate into mesodermal tissue such as various mesenchymal tissues or nerve tissues, they are useful as a source of cells for tissue regenerating therapy to congenital diseases, degenerative diseases, and injuries. For instance, as for disease or pathology to be the object of the therapeutic agent or treating method of the present invention, bone destruction due to degenerative disease such as dysostosis, fracture, rheumatoid arthritis; cartilage destruction due to rheumatoid arthritis or osteoarthritis, or muscular disease due to congenital disease such as dystrophy or acquired disease such as myositis; myocardial disease due to myocardial infarction or cardiomyopathy, brain disorder such as brain infarction or Parkinson disease; injury such as spinal cord damage, or vascular disease due to arteriosclerosis or connective tissue disease. Moreover, plastic surgery such as breast augmentation and the like is encompassed in the therapeutic agent or treating method of the present invention for convenience. In cell therapy using MOMCs

or MOMCs treated by inducing differentiation, there are considerable advantages over currently proposed regenerative treatment using tissue-specific stem cells and ES cells. In other words, as a large number of monocytes can be obtained from patients by collecting their blood, a minimally invasive procedure, circulating monocytes could be a relatively easy source of autologous cells. Furthermore, the generation of MOMCs from monocytes is technically easy and quick, and the ethical dilemma of using ES cells can be bypassed.

0024

Example

The present invention will be explained in detail in the following, but the technical scope of the present invention will not be limited to these examples.

[Methods and materials]

Example 1 (MOMC cultures)

PMBCs were isolated from heparinized venous blood obtained from healthy adult by Lymphoprep (Nycomed Pharma AS) density gradient centrifugation. All blood samples were obtained after the subjects gave their written informed consent, approved by the Institutional Review Boards. Isolated cells were washed twice with phosphate-buffered saline (PBS) and suspended in low-glucose DMEM supplemented with 10% heat-inactivated FBS (JRH Biosciences). PMBCs were cultured at a density of $2 \times 10^6/\text{ml}$ on plastic plates coated with fibronectin without any additional growth factors at 37°C with 5% CO_2 , in a humidified atmosphere. Three days after the culture, non-adherent cells were removed. The medium was changed to a fresh one every three days and the cells were cultured for up to 4 weeks. After 7–10 days of culture, the adherent cells were collected as MOMCs and used in the following

assays or were removed on new fibronectin-coated plates and maintained in the same culture condition for up to 10 passages.

0025

To examine the origin of MOMCs, PBMCs depleted of CD14⁺, CD34⁺ or CD105/endoglin/SH2⁺ cells were cultured on fibronectin-coated plates for 7 days. The depletion of CD14⁺ or CD34⁺ cells was performed by using an anti-CD14 or anti-CD34 monoclonal antibody coupled to magnetic beads (DynaBeads) followed by magnetic separation according to the manufacturer's protocol. CD105⁺ cell-depleted PBMCs were prepared by incubating PBMCs with anti-CD105 mAb (Immunotech) and subsequently with goat anti-mouse IgG antibody coupled to magnetic beads (DynaBeads). Mock-treated PBMCs incubated with isotype-matched mouse mAb and bead-conjugated anti-mouse IgG antibody were also prepared as a control. The proportion of CD14⁺ cells in the CD14⁺ cell-depleted PBMC fraction was consistently <0.5%, and the proportions of CD34⁺ cells in the CD34⁺ cell-depleted fraction and of CD105⁺ cells in the CD105⁺ cell-depleted fraction were <0.01% by flow cytometry. The number of attaching cells per cm² was counted and the results were expressed as the ratio to the untreated PBMC culture.

0026

Some experiments were performed to separate circulating CD14⁺ monocytes and CD14⁻ cells from PBMCs by using anti-CD14 mAb-coupled magnetic beads (CD14 MicroBeads; Miltenyi Biotech) followed by MACS column separation according to the manufacturer's protocol. Flow cytometric analysis revealed that monocyte and CD14⁻ cell fractions contained >98% and <0.5% CD14⁺, respectively. Monocytes were labeled with PKH67 green (Sigma) and cocultured with unlabeled CD14⁻ cells (ratio of 1:4) on fibronectin-coated

or -uncoated plastic plates for 7 days. PKH67-labeled monocytes were also cultured alone on fibronectin-coated plates.

0027

Example 2 (Preparation of macrophages and dendritic cells)

Macrophages were prepared by culturing adherent PMBCs on plastic plates in M199 medium supplemented with 20% FBS and 4 ng/ml M-CSF (R&D Systems) for 7 days (Differentiation, 65, 287-300, 2000). Mature monocyte-derived dendritic cells were obtained from plastic adherent PBMCs by maturation using a series of culture conditions (J Immunol Methods, 196, 121-135, 1996). Briefly, adherent cells were cultured in RPMI 1640 supplemented with 10% FBS containing 50 ng/ml GM-CSF and 50 ng/ml IL-4 (all from PeproTech) for 7 days. Next, the immature dendritic cells were incubated with 50 ng/ml TNF- α (PeproTech) for 3 days. Flow cytometric analysis revealed that the macrophage fraction contained 98% or more of CD14⁺CD80⁺ cells and the dendritic cell fraction contained 95% or more of CD83⁺HLA-DR⁺ cells and less than 1% of CD14⁺ cells.

0028

Example 3 (Cell lines)

Primary cultures of human dermal fibroblasts were established from dermal biopsies of healthy donors and maintained in low-glucose DMEM supplemented with 10% FBS. Primary human myoblasts were prepared by culturing muscle biopsies from patients that are histologically normal even being clinically suspected as myositis (J Cell Biol., 144, 631-643, 1999). The human osteosarcoma cell line MG-63, the rhabdomyosarcoma cell line RD, and the chondrosarcoma cell line OUMS-27 were obtained from Health Science Research Resources Bank of Japan (Osaka, Japan) and maintained in low-glucose DMEM supplemented with 10% FBS.

0029

Example 4 (In vitro differentiation of MOMCs into mesenchymal cells)

MOMCs which were either freshly generated from PBMCs, cultured for several passages or cryo-preserved, were moved on new fibronectin-coated plastic plates or chamber slides and grown to semi-confluence in high-glucose DMEM supplemented with 10% FBS (Hyclone Laboratories). Next, the cells were then cultured under conditions known to induce MSCs to differentiate into various mesenchymal cell types cited in the following (Science, 284, 143-147, 1999; J Cell Biol., 144, 631-643, 1999; J Cell Biochem., 64, 295-312, 1997; Muscle Nerve, 18, 1417-1426, 1995; Tissue Eng., 4, 415-428, 1998; Arthritis Rheum., 44, 85-95, 2001; J Biol Chem., 275, 9645-9652, 2000). Monocytes, macrophages, and dermal fibroblasts newly isolated from PBMCs were treated under identical conditions as controls.

0030

As for osteogenesis, the adherent cells were cultured in Osteogenesis induction medium (Clonetics) containing 100 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ M ascorbic acid. The medium was changed twice a week for 3 weeks.

0031

As for myogenesis, the adherent cells were treated with 10 μ M 5-azacytidine (Sigma) for 24 hours. The cells were washed with PBS and cultured in a medium containing 5% horse serum (Life Technologies), 50 mM hydrocortisone (Sigma) and 4 ng/ml basic fibroblast growth factor (Sigma). The medium was changed twice a week for 3 weeks.

0032

As for chondrogenesis, the adherent monolayer cells were cultured for 3 weeks in serum-free medium in the presence of

TGF- β (R & D systems), which was added to the culture medium every other day so that the final concentration become 10 ng/ml.

0033

As for adipogenesis, the cells were incubated with adipogenic induction medium supplemented with 1 μ M dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 μ g/ml insulin, and 100 mM indomethacin (all from Sigma). After 72h, the medium was changed to maintenance medium supplemented with only 10 μ g/ml insulin and rested for 24 hours. The cells were treated three times with adipogenic induction medium and maintained in the maintenance medium for an additional week.

0034

Example 5 (In vitro differentiation of MOMCs into myocardial cells)

MOMCs were cocultured with cultured myocardial cells derived from Wistar rat fetus for 8 days. The cultured cells were fixed with 4% paraformaldehyde, and then immunostained (DAB staining) with human-specific anti-nestin antibody (Chemicon).

0035

After labeling cell membrane with fluorescent PKH67 (Sigma), MOMCs were cocultured with Wistar rat cultured myocardial cells for 7 to 10 days. The cultured cells were fixed with 4% paraformaldehyde, and double fluorescently immunostained with anti-Nkx2.5 antibody, anti-eHAND antibody (Santa Cruz), and anti-CD45 antibody (DAKO).

0036

MOMCs were cocultured with Wistar rat cultured myocardial cells for 3, 6, 9, and 12 days, and mRNA was extracted. Human specific PCR primers (TGACAAGAACGATCTGAGAG, CAGGTTCTTGTAGTCCAAGT) to myocin light chain (MLC2v) being a

myocardial cell structural protein were constructed and RT-PCR was performed.

0037

Example 6 (In vitro differentiation of MOMCs into neurons)

MOMCs were cocultured with Wistar rat cultured neurons for 8 days. The cultured cells were fixed with 4% paraformaldehyde, and then immunostained with human-specific anti-nestin antibody (DAB staining).

0038

After labeling cell membrane with fluorescent PKH67, MOMCs were cocultured with Wistar rat cultured neurons for 4 days. The cultured cells were fixed with 4% paraformaldehyde, and double fluorescently immunostained with anti-NeuroD antibody (Santa Cruz) and anti-CD45 antibody (DAKO).

0039

MOMCs were cocultured with Wistar rat cultured neurons for 3 days. The cultured cells were fixed with 4% paraformaldehyde, and double fluorescently immunostained with human-specific anti-nestin antibody and anti-Neurogenin 2 antibody (Chemicon).

0040

After labeling cell membrane with fluorescent PKH67, MOMCs were cocultured with Wistar rat cultured neurons for 9 to 10 days. The cultured cells were fixed with 4% paraformaldehyde, and double fluorescently immunostained with anti-Hu antibody and anti-NeuN antibody (Chemicon).

0041

Example 7 (In vitro differentiation of MOMCs into endothelial cells)

MOMCs were cultured in endothelial cell maintenance medium EBM-2 (Clonetics) for 7 to 10 days as adherent cells. The cultured

cells were fixed with 10% neutral buffered formalin, and immunostained (DAB staining) with anti-CD34 antibody (Calbiochem-Novabiochem), anti-vWF antibody (Dako), anti-eNOS antibody (Becton-Dickinson) and anti-VEGFR2/KDR/Flk-1 antibody (Sigma).

0042

RNA was extracted from MOMCs cultured in EBM-2 medium for 7 days, and reverse-transcribed to cDNA. PCR was performed by using specific primers to Flt-1, VEGFR/2/KDR/Flk-1, CD31, CD144, vWF and CD34, CD45, CD14, GAPDH, expressed in endothelial cells. MOMCs before inducing differentiation and RNA derived from cultured umbilical vein endothelial cell (HUVEC) were used as control.

0043

Example 8 (Flow cytometry analysis)

Fluorescent cell staining was performed with the following steps. The adherent cells were detached from the plastic plates by incubation with 2 mM EDTA on ice, and blocked with normal mouse or rat serum for 10 min at under 4°C. The cells were stained with the following mouse mAbs or rat anti-Sca-1 mAb (Cedarlane Laboratories), which were either unconjugated or conjugated to FITC, phycoerythrin (PE) or PC5: anti-HLA-DR antibody, anti-CD11c antibody (BD Pharmingen), anti-CD11b/Mac-1 antibody, anti-CD14 antibody, anti-CD29 antibody, anti-CD34 antibody, anti-CD44 antibody, anti-CD83 antibody, anti-CD105/endothelin/SH2 antibody, anti-CD117/c-kit antibody (Immunotech), anti-CD34 antibody, anti-CD133 antibody (Miltenyi Biotec), anti-HLA class I antibody, anti-HLA-DR antibody, anti-CD31/PECAM-1 antibody, anti-Flt-1/VEGFR1 antibody, anti-Flk-1/VEGFR2 antibody (Sigma), anti-CD40 antibody, anti-CD54 antibody, anti-CD80 antibody, anti-CD86 antibody (Ancell), anti-CD144/VE-cadherin antibody,

or anti-type I collagen antibody (Chemicon International). When unconjugated mAbs were used, goat anti-mouse antibody or rat IgG F (ab')₂ antibody conjugated to FITC or PE (Immunotech) was used as a secondary antibody. For intracellular staining, the cells were permeabilized and fixed by using IntraPrep™ permeabilization reagent (Immunotech). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) by using the Cell Quest software. Visualized cells were identified by gating on forward and side scatters, and the data are shown as logarithmic histograms or dot-plots.

0044

Example 9 (Immunohistochemistry)

Slides were coated with monocytes, macrophages, or dendritic cells by using a cytospin technique, and the remaining cell types were cultured on fibronectin-coated chamber slides, except for samples to be used for fibronectin staining, for which type I collagen-coated slides were used instead. The cells were fixed with 10% formalin, and the endogenous peroxidase activity was suppressed with 3% peroxide for 5 min. Slides were incubated for 30 min with one of the following mouse mAbs, or rat anti-Sca-1 mAb: anti-CD45 antibody, anti-vimentin antibody, anti-skeletal muscle-specific actin antibody (SkM-actin) (Dako), anti-CD34 antibody (Calbiochem-Novabiochem), anti-type I collagen antibody (Chemicon), anti-type III collagen antibody, anti-fibronectin antibody (Sigma), anti-type II collagen antibody (ICN Biomedicals) or anti-skeletal muscle-specific myosin heavy chain antibody (SkM-MHC) (Zymed Laboratories). The slides were then further incubated with biotin-labeled anti-mouse antibody and anti-rat IgG antibody. The antibody-biotin conjugates were detected with a streptavidin-horseradish peroxidase complex (Nichirei) applied

for 10 min at room temperature by using 3,3'-diaminobenzidine as the substrate. Nuclei were counterstained with hematoxylin. The negative controls were cells incubated with normal mouse- or rat-IgG antibody (DAKO) instead of the above-mentioned primary antibody.

0045

Fluorescence double-staining was performed as follows. The cells were fixed with 4% paraformaldehyde, and incubated with goat anti-PEBP2 α A antibody or anti-Sox9 polyclonal antibody (Santa Cruz), followed by incubation with AlexaFluor R568 goat-specific IgG antibody (Molecular Probes) and then with FITC-conjugated mouse anti-CD45 mAb (Dako). Similarly, the cells were stained with mouse anti-MyoD antibody (Dako) or anti-peroxisome proliferation-activated receptor γ . (PPAR γ gene) mAb (Santa Cruz), followed by incubation with tetramethylrhodamine isothiocyanate isomer R-labeled mouse-specific IgG antibody (Dako) and subsequently with FITC-conjugated anti-CD45 mAb. The cells were examined with a confocal laser fluorescence microscope (LSM5 PASCAL; Carl-Zeiss). To enumerate the proportion of cells staining positive for a given marker, at least 300 cells per culture were evaluated with a low magnification.

0046

Example 10 (Uptake of acetylated LDL (Ac-LDL))

The adherent cells were cultured with 2.5 μ g/ml Dil-Ac-LDL (Molecular Probes) for 1 hour, and Ac-LDL uptake was evaluated by flow cytometry.

0047

Example 11 (Alkaline phosphatase staining)

The cells were fixed with 10% formalin and subsequently incubated in a solution containing 0.2 mg/ml naphthol AS-TR phosphate

and 0.5 mg/ml Fast Red RC (all from Sigma) for 10 min.

0048

Example 12 (Intracellular calcium detection)

To evaluate intracellular calcium deposits, the cells were fixed with 10% formalin and stained with 2% alizarin red S (Sigma) for 3 min, followed by extensive wash with distilled water. The intracellular calcium concentration was measured by using a commercially available kit (Sigma) (J Biol Chem., 275, 9645-9652, 2000). The protein content in cell extract was also measured by using the Bradford protein assay kit (Bio-Rad Laboratories) by using bovine serum albumin as a standard. The calcium concentration was expressed as microgram per microgram of protein content.

0049

Example 13 (Oil-red-O staining)

The cells were fixed with 0.2% glutaraldehyde for 5 min, washed with 60% isopropanol, and covered with 0.1% Oil-red-O (Sigma) for 10 min. After washing with 60% isopropanol and subsequently with distilled water, the cells were counterstained with hematoxylin.

0050

Example 14 (Transmission electron microscopy)

Cultured MOMCs were immediately fixed with 2.5% glutaraldehyde, post-fixed with 2% osmium tetroxide, dehydrated in a series of ethanol and propylene oxide, and embedded in Epoxy resin. The cells were thin-sectioned on a LKB ultratome with a diamond knife. Sections in the range of gray to silver were collected on 150-mesh grids, double-stained with uranyl acetate and lead citrate, and examined under a JEOL-1200 EXII electron microscope (Jeol).

0051

Example 15 (Cell proliferation studies)

Proliferating MOMCs were detected by BrdU-labeling as described previously (Blood, 71, 1201-1210, 1988). It is explained briefly in the following. MOMCs were cultured in the presence of 10 μ M BrdU (Sigma) for 2 hours before staining. After a 30-minute fixation in Carnoy's fixative (methanol/acetic acid) at -20°C, the cells were air-dried, treated with 2N-HCl for 1 hour to denature DNA, and then neutralized with 0.1 M borate (pH 8.5) for 10 minutes. The cells were then incubated with mouse anti-BrdU mAb (Chemicon International), followed by biotin-streptavidin-peroxidase complex staining. Nuclei were counterstained with hematoxylin. Negative controls were the cells incubated with isotype-matched mouse control mAb instead of the above-mentioned primary antibody. Apoptotic cells were detected by incubating unfixed cells with propidium iodide (Sigma) for 30 min, and observed under a fluorescent microscope.

0052

For cell-division studies, the cells were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) as described previously (J Exp Med., 183, 2313-2328, 1996). CFSE-labeled monocytes were cocultured with unlabeled CD14⁺ cells on fibronectin-coated plates for 1, 3 and 5 days, and the adherent cells were harvested and stained with PE5-labeled anti-CD14 mAb. CFSE-labeled MOMCs were also cultured for 1, 3 and 5 days. The color intensity of CFSE labeling was evaluated by flow cytometry.

0053

Example 16 (RT-PCR)

Total RNA was extracted from MOMCs that had or had not been induced to differentiate by using the RNeasy kit (Qiagen).

Total RNA was also extracted from peripheral blood CD14⁺ monocytes and macrophages, dendritic cells, dermal fibroblasts, myoblasts, and various cultured cells including osteosarcoma, rhabdomyosarcoma, and chondrosarcoma. Human muscle- and fat tissue-derived total RNAs were purchased from Clontech Laboratories. Single-strand cDNA was synthesized from the total RNA by using Molony murine leukemia virus reverse transcriptase (Takara) with oligo-dT as a primer. The cDNA (equivalent to 50 ng total RNA) was then subjected to PCR amplification by using various specific primers listed in Table 1, shown by SEQ ID NOs: 3 to 34. The PCR products were resolved by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide.

0054

Table 1

Gene	Primer sequences	Product size (bp)
Osterix	Sense: 5'-CTTGTGCCTGATACCTGCACT-3'	470
	Antisense: 5'-TCACTCTACCTGACCCGTCATC-3'	
Bonesialoprotein II	Sense: 5'-AAACGGCACCAGTACCAACA -3'	394
	Antisense: 5'-GCCATCGTAGCCTTGTCCTT-3'	
Osteocalcin	Sense: 5'-GGCAGCGAGGTAGTGAAGAGAC-3'	257
	Antisense: 5'-GGCAAGGGGAAGAGGAAAGAAG-3'	
SkM-MHC	Sense: 5'-ATAGGAACACCCAAGCCATC-3'	599
	Antisense: 5'-TTTGCCTAGACCCTTGACAG-3'	
Myogenin	Sense: 5'-TGGCCTTCCCAGATGAAACC-3'	452
	Antisense: 5'-GCATCGGGAAGAGACCAGAA-3'	
$\alpha 1$ (II) collagen	Sense: 5'-TTCAGCTATGGAGATGACAATC-3'	472
	Antisense: 5'-AGAGTCCTAGAGTGACTGAG-3'	
$\alpha 1$ (X) collagen	Sense: 5'-AATCCCTGGACCGGCTGGAATTC-3'	267
	Antisense: 5'-TTGATGCCTGGCTGTCCTGGACC-3'	
PPAR γ	Sense: 5'-AGGAGCAGAGCAAAGAGGTG-3'	474
	Antisense: 5'-AGGACTCAGGGTGGTTTCAGC-3'	
aP2	Sense: 5'-TATGAAAGAAGTAGGAGTGGGC-3'	290
	Antisense: 5'-CCACCACCAGTTTATCATCCTC-3'	
CD34	Sense: 5'-CCTCCCAAGTTTTAGGACAA-3'	362
	Antisense: 5'-CAGCTGGTGATAAGGGTTAG-3'	
CD45	Sense: 5'-AACCTGAAGTGATGATTGCTG-3'	500
	Antisense: 5'-TACCTCTTCTGTTTCCGCAC-3'	
CD14	Sense: 5'-CTGCGTGTGCTAGCGTACTC-3'	655
	Antisense: 5'-CGTCCAGTGTGAGGTTATCC-3'	
Cbfa1/Runx2	Sense: 5'-GTCTTACCCCTCCTACCTGA-3'	183
	Antisense: 5'-TGCCTGGCTCTTCTTACTGA-5'	
MyoD	Sense: 5'-CCTAGACTACCTGTCCAGCATC-3'	365
	Antisense: 5'-GGCGGAAACTTCAGTTCTCC-3'	
Sox-9	Sense: 5'-CCCGATCTGAAGAAGGAGAGC-3'	380
	Antisense: 5'-GTTCTTCACCGACTTCCTCCG-3'	
GAPDH	Sense: 5'-TGAACGGGAAGCTCACTGG-3'	307
	Antisense: 5'-TCCACCACCCTGTTGCTGTA-3'	

0055

Example 17 (Statistical analysis)

All comparisons were tested for statistical significance by using Mann-Whitney U test.

0056

[Results]

Example 18 (Generation of MOMCs)

When PBMCs were cultured on fibronectin-coated plates in DMEM supplemented only with 10% FBS, a subset of cells immediately attached to the plates. Small clusters of round cells developed within 24 hours, and cell processes extended from them. After 5 days of culture, adherent cells with a fibroblast-like morphology made their appearance. Over the ensuing 3 days, the fibroblast-like cells became the predominant cell type in the culture (Fig. 1a). The fibroblast-like cells were frequently detected around the clusters (Fig. 1b). The number of fibroblast-like cells increased slowly until around Day 14. After this time, the proliferating ability gradually stopped but the cells survived for up to 4 weeks. When 10^8 PBMCs obtained from 50 donors were cultured, 0.3 to 1.0×10^7 adherent cells were obtained at Day 7. From flow cytometric analysis, the cells harvested on Day 7 showed to comprise a single phenotype (95% or more were homogeneous) and to be positive for CD14, CD45, CD34, and type I collagen (Fig. 2). This phenotype is unique and distinct from that of known adherent cells of peripheral blood origin, including monocytes/macrophages ($CD14^+$, $CD45^+$, $CD34^-$ and $type\ I\ collagen^-$), endothelial progenitors ($CD14^-$, $CD45^-$, $CD34^+$ and $type\ I\ collagen^-$) (Science, 275, 964-967, 1997), and mesenchymal progenitors ($CD14^-$, $CD45^-$, $CD34^-$, $type\ I\ collagen^+$) (Arthritis Res., 2, 477-488, 2000). Cells obtained

from at least 50 donors showed the same phenotype. After the cells were moved on new fibronectin-coated plates on Day 7 and cultured under the same conditions, nearly all the cells adopted an elongated fibroblast-like morphology (Fig. 1c). These cells could be expanded up to 5 passages, and the cell proliferation was likely to be most active just after the passage. However, after 5 passages, the cell proliferation speed became gradually slow, and the proliferation ability disappeared after 8 passages. The cells did not differentiate naturally into mature mesenchymal cells during the culture when no particular treatment was performed. However, when cells were inoculated at a high confluency, cells with plural nuclei appeared at a very low frequency. These fibroblast-like cells obtained from PBMCs from a culture in vitro, were named MOMC from the following findings.

0057

By electron microscopic examination, it was revealed that MOMCs had a spindle shaped morphology and contained a number of cytoplasmic organelles (Figs. 1d-g). Multiple primary lysosomes, cell surface projections like pseudopodia, and labyrinth-like endocytic vesicles are specific observations that are also found in macrophages and other phagocytes. MOMCs also had prominent bundles of intermediate filaments, well-developed secondary lysosomes, and elongated and branching mitochondria, which are characteristics frequently seen in cells of mesenchymal origin. Small lipid droplets were observed in almost all MOMCs. In addition, structures similar to rod-shaped micro-tubulated bodies, which are specific to endothelial cells, were frequently detected. These ultrastructural findings represented mixed features of phagocytes, and mesenchymal and endothelial cells.

0058

Example 19 (Origin of MOMCs in circulation)

The adherent cells obtained in the PBMC culture on fibronectin-coated plates were serially examined for their surface expression of CD14 and CD34 by flow cytometry (Fig. 3a). The majority of cells attached to the plates at 1 hour were CD14⁺ and CD34⁻, but CD34 expression gradually up-regulated on the adherent CD14⁺ cells. Nearly all the adherent cells were positive for both CD14 and CD34 after 7 days in culture. As peripheral blood contained CD34⁺ endothelial progenitors (Science, 275, 964-967, 1997) and CD105/endoglin/SH2⁺ mesenchymal progenitors (Science, 284, 143-147, 1999; Arthritis Res., 2, 477-488, 2000; Biochem Biophys Res Commun., 265, 134-139, 1999), the present inventor examined the effect of depleting PBMCs of cells positive for CD14, CD34, or CD105 on the in vitro induction of MOMCs. As shown in Fig. 3b, the appearance of MOMCs was almost completely inhibited by the depletion of CD14⁺ monocytes, whereas depletion of CD34⁺ or CD105⁺ cells showed no effect. To further confirm that MOMCs originated from circulating monocytes, CD14⁺ monocytes were isolated from PBMCs, labeled with PKH67 and cultured with unlabeled CD14⁻ cells on fibronectin-coated plates. As shown in Fig. 3c, PKH67-labeled monocytes expressed CD34 after one week of the culture. Together, these findings indicate that MOMCs originates from circulating CD14⁺ monocytes. However, non-adherent cells collected on Day 3 contained a significant proportion of CD14⁺ cells, which suggests that a subset of CD14⁺ monocytes can attach to fibronectin and differentiate into MOMCs.

0059

When PKH67-labeled monocytes were cultured alone on fibronectin, only a few cells became fibroblast-like and CD34 expression was not up-regulated at Day 7 (Fig. 3c). In this case,

it was sufficient to add culture supernatant of CD14⁻ cells to induce CD34 expression in monocytes. Furthermore, when coculturing CD14⁻ cells with PKH67-labeled monocytes on plates that are not coated with fibronectin, the number of cells attached to the plates on Day 7 was low, and these adherent cells did not express CD34 (Fig. 3c). From these results, it is likely that the differentiation from circulating monocytes into MOMCs requires soluble factor (1 or more) derived from CD14⁻ cells in peripheral blood and binding to fibronectin.

0060

To evaluate whether CD14⁺ monocytes proliferate during MOMC differentiation, CD14⁺ monocytes were labeled with CFSE and cocultured with unlabeled CD14⁻ cells on fibronectin. Adherent cells were serially harvested and examined for color intensity of CFSE labeling and CD14 expression by flow cytometry (Fig. 3d). Nearly all fibronectin-attached monocytes proliferated mainly during the first 24 hours of culture, and proliferated slowly later in culture. Adherent cells were almost exclusively CFSE-labeled CD14⁺ monocytes, and the expansion of adherent cells from the CD14⁻ cell fraction was not observed, suggesting that generation of MOMCs did not occur as a result of the growth of specific precursors in the mixed CD14⁻ cell population.

0061

Example 20 (Phenotype of MOMCs)

Expression of various cell surface molecules and intracellular molecules of MOMCs was examined by flow cytometry and immunohistochemistry (Figs. 2 and 4), and compared the protein expression profile with that of monocytes, macrophages, and dendritic cells (Table 2). MOMCs expressed hematopoietic and monocyte lineage marker genes (CD45, CD14, CD13, CD11b, CD11c,

and CD64), but did not express dendritic cell marker genes (CD1a and CD83). The expression of HLA Class I, HLA-DR, and costimulatory molecules (CD40 and CD86) on MOMCs strongly suggests that MOMCs have an ability to induce antigen-specific T cell activation as antigen-presenting cells. MOMCs expressed hematopoietic stem/endothelial cell marker gene CD34, and mesenchymal stem/endothelial cell marker gene CD105/endoglin/SH2 (Biochem Biophys Res Commun., 265, 134-139, 1999). Moreover, MOMCs expressed stem cell marker gene Sca-1, but did not express different stem cell marker genes CD117/c-kit and CD133. Further, MOMCs were positive for the endothelial marker gene CD144/VE-cadherin and Flt-1/VEGFR1, while Flk-1/VEGFR2 and vWF expressions were not observed. MOMCs were also positive for type I and III collagen, fibronectin, and vimentin, which are extracellular matrix proteins typically produced by cells of mesenchymal origin. These protein expression profiles did not change for up to 5 passages. MOMCs showed distinct phenotypes from that of monocytes and monocyte-derived phagocytes. In particular, the expression of stem cell marker genes (CD34, Sca-1 and CD105), endothelial marker genes (CD144/VE-cadherin and Flt-1/VEGFR1), and mesenchymal marker genes (type I and III collagen, and fibronectin) is unique characteristic of MOMCs. Therefore, it can be recognized that MOMCs are cells with phenotypes of phagocytes, endothelial cells, mesenchymal cells and stem cells.

0062

Table 2

	Monocytes	Macrophages	Dendritic cells	MOMCs
CD45	++	++	+	+
Monocyte markers				
CD14	++	++	–	++
CD13	+	+	+	++
CD11b/Mac-1	++	+	++	+
CD11c	+	+	+	+
CD64	+	+	–	+
Dendritic cell markers				
CD1a	–	–	–/++ ^A	–
CD83	–	–	++	–
HLA molecules				
HLA class I	++	+++	+++	++
HLA-DR	++	++	++	++
Costimulatory molecules				
CD40	+	+	++	+
CD80	–	++	++	–
CD86	+	+	+++	+
Adhesion molecules				
CD29	+	+	+	+
CD44	++	++	++	+ / ++ ^A
CD54	+	+	++	+
Stem cell/progenitor markers				
CD34	–	–	–	+
CD105/engoglin/SH2	–	–	–	+
CD117/c-kit	–	–	–	–
CD133	–	–	–	–
Sca-1	–	–	–	+ / ++ ^A
Endothelial cell markers				
CD31	+	+	+	+
CD144/VE-cadherin	–	–	–	+
Flt-1/VEGFR1	–	–	–	+
Flk-1/VEFR2	–	–	–	–
vWF ^A	–	–	–	–
Ac-LDL	+	++	–	++
Mesenchymal cell markers				
Type I collagen	–	–	–	+
Type III collagen	–	–	–	+
Fibronectin	–	–	–	+
Vimentin	+	+	+	++

0063

Example 21 (Proliferating ability of MOMCs)

MOMCs seemed to increase during culture. To investigate whether this observation is due to cell division, the proportion of dividing cells in MOMCs was evaluated serially by BrdU staining (Fig. 5a). Nearly half of the adherent cells were stained with BrdU after 1 day from the passage, but the number of BrdU⁺ cells decreased significantly at Day 5. The proportion of BrdU⁺ cells to all adherent cells were calculated at Day 1, 3, 5, 7 and 10, and the proportion of BrdU⁺ cells were at the maximum at Day 1 during culture, and decreased chronologically afterwards. Cells positive for propidium iodide staining were less than 1% at all time points. By investigating using CFSE labeling, it appeared that MOMCs divided actively and synchronously after the passage and no subset of the cells proliferated predominantly (Fig. 5c). These findings suggest that MOMCs have proliferating ability during culture, and that mainly proliferate just after the passage.

0064

Example 22 (In vitro differentiation of MOMCs into mesenchymal cell lineages)

As MOMCs had some morphologic and phenotypic properties of mesenchymal cells, the present inventors hypothesized that MOMCs can be induced to differentiate into some mesenchymal lineages. To confirm this hypothesis, MOMCs were cultured under various conditions known to induce differentiation of MSCs into bone, skeletal muscle, cartilage and fat.

0065

MOMCs treated with the osteogenic induction procedure underwent a change in their morphology from spindle-shaped to

cylinder-like form. It was observed that almost every adherent cell formed calcium deposits, by alizarin red staining (Fig. 6a), and these adherent cells expressed alkaline phosphatase (Fig. 6b). The intracellular calcium content was significantly increased during this process (Fig. 6c). After osteogenic induction, MOMCs expressed mRNAs for bone sialoprotein II produced by mature osteocytes and osteocalcin (Calcif Tissue Int. 62, 74-82, 1998) and for bone-specific transcription factor osterix (Cell, 108, 17-29, 2002). On the other hand, CD34, CD45, and CD14 expression was lost after this induction treatment (Fig. 6d).

0066

When MOMCs were treated with 5-azacytidine and cultured under a condition inducing differentiation into muscle for 3 weeks, the cells became elongated, but no cells like myocytes with plural nuclei appeared. At this time point, expression of SkM-actin and SkM-MHC was induced in 45-60% of the adherent cells, depending on the sample (Fig. 6e and 6f). By RT-PCR, mRNAs for the muscle-specific transcription factor myogenin and SkM-MHC were detected after induction (Fig. 6g). The expression of CD34, CD14, and CD45 was reduced but not lost. Immunohistochemical analysis revealed that CD34 was expressed in nearly all adherent cells, but CD14 and CD45 were expressed in cells that did not express SkM-MHC (data not shown). The expression CD34 was also detectable in cultured myoblasts and muscle tissue, and even in a rhabdomyosarcoma cell line, consistent with the expression of CD34 in a subset of primitive muscle cells (J Cell Biol., 150, 1085-1100, 2000).

0067

To induce cartilage formation, MOMCs were cultured in

micromass suspension in the presence of TGF- β 1, which is a standard method to induce chondrocyte differentiation in MSC (Science, 284, 143-147, 1999; Tissue Eng., 4, 415-428, 1998; Arthritis Rheum., 44, 85-95, 2001). However, MOMCs died within 1 week even in culture according to several different protocols by droplet-micromass on plates, or pelette-macromass in conical tube. Therefore, monolayer MOMCs were cultured in the presence of TGF- β 1 for 3 weeks. As it is shown in Fig. 6h, type II collagen typical of articular cartilage, was weakly expressed in untreated MOMCs, but its expression was markedly up-regulated after induction treatment. Results of RT-PCR further demonstrated the up-regulated expression of chondrocyte-specific type II and type X collagen after the induction treatment (Fig. 6i). The expression of CD45 and CD14 was lost, but CD34 expression was retained after the induction treatment. On the other hand, CD34 was also expressed in a chondrosarcoma cell line.

0068

Electron microscopic examination revealed small lipid droplets in MOMCs (Figs. 1d, f and g). After the induction treatment, lipid vacuoles appeared and increased over time in both size and number. These lipid vacuoles were stained with Oil-red-O (Fig. 6j). From this induction treatment, 50 to 80% of the adherent cells were committed to this lineage, depending on the sample. mRNAs for PPAR γ genes, and mRNAs for the fatty acid binding protein aP2 were weakly expressed in MOMCs, but the expression of these genes were markedly up-regulated after the above-mentioned induction treatment (Fig. 6k). Expression of mRNAs for CD45 and CD14 was lost, but CD34 expression was retained after the induction treatment. On the other hand, CD34 was also expressed in fat tissues.

0069

The differentiation into mesenchymal cells was observed in MOMCs that were freshly generated from PBMCs, cultured for up to five passages or cryo-preserved. In addition, MOMCs obtained from 5 donors showed similar differentiation potential. Two strains of human dermal fibroblasts, which are mature mesenchymal cells as well as freshly isolated CD14⁺ monocytes and macrophages, were also cultured under the identical induction conditions. After 3 weeks, the dermal fibroblasts did not show any differentiation tendency in these conditions, although the cells appeared healthy. After 1 week, circulating monocytes and macrophages subjected to these culture conditions detached from the plates without apparent differentiation. Lineage-specific differentiation was observed in more than half of the adherent cells after 3 weeks of culture. But the number of the adherent cells decreased by 20 to 50% from the initial number of cells, suggesting that most MOMCs were detached during the induction process.

0070

To investigate whether the differentiation into a certain lineage is specific to various induction treatments, cultures from each treatment were cross-stained with alizarin red, Oil-red-O, and were also immunostained with SkM-MHC or type II collagen. These cells were positive only for the staining specific to the intended lineage, and negative to all other stainings (data not shown). Furthermore, to investigate the possibility that some subsets differentiate into lineage other than the intended lineage in an in vitro assay, cells treated with differentiation induction for 3 weeks were subjected to high-sensitive RT-PCR, to amplify the transcripts of plural genes wherein the expression is limited

to osteogenic (osteocalcin), myogenic (SkM-MHC), chondrogenic (type II collagen), or adipogenic (aP2) lineage. Expression of lineage-specific gene was specific to the intended lineage (data not shown), suggesting that the differentiation was exhibited specifically to the performed treatment.

0071

To exclude the possibility that the differentiated mesenchymal cells were derived from cells with differentiation potential as MSC contaminated into MOMC fractions, CD14⁺ cells were positively purified from the MOMCs by using the MACS separation system before the induction treatment. As expected, the differentiation into the osteogenesis, myogenesis, chondrogenesis, and adipogenesis was observed as for the unselected MOMCs. In addition, the depletion of cells expressing CD34 or CD105/endoglin/SH2 at the initiation of PBMC cultures did not affect the differentiation into mesenchymal cells. Further the expression of lineage-specific transcription factors, Cbfa1/Runx2 (Cell, 89, 755-764, 1997), MyoD (Front Biosci., 5, D750-767, 2000), Sox-9 (Osteoarthritis Cartilage, 8, 309-334, 2000), and PPAR γ (J Biol Chem., 276, 37731-37734, 2001), in MOMCs after 1 week of induction treatment, was examined. As at this time point, as MOMCs were expressing CD45, MOMCs that underwent osteogenic, myogenic, chondrogenic, or adipogenic induction treatment were double-stained, to investigate the expression of CD45 and individual transcription factors. As shown in Fig. 7, MOMCs that underwent osteogenic differentiation for 1 week, expressed CD45 in cell membrane and cytoplasm, and Cbfa1/Runx2 in nucleus. Similarly, simultaneous expression of CD45/MyoD and CD45/Sox-9 was observed in MOMCs that underwent induction of myogenic and chondrogenic differentiation for 1 week. PPAR γ

was weakly expressed in the nuclei of untreated MOMCs, but after 1 week of adipogenic induction, the PPAR γ expression increased and the CD45 expression decreased. These findings suggest that CD45⁺ hemopoietic cells underwent lineage-specific differentiation under specific conditions inducing differentiation. The expression of these lineage-specific transcription factors, except PPAR γ , was lost at 3 weeks of the induction treatment as determined by immunohistochemistry and RT-PCR (data not shown). These findings suggest that MOMCs express transiently the above-mentioned lineage-specific transcription factors in the primary differentiation step of the induction treatment.

0072

Example 23 (In vitro differentiation of MOMCs into myocardial cells)

Nestin (Brown), a marker expressing in nerve and myocardial progenitors, was expressed in MOMCs at Day 8 of coculture, and binding with surrounding rat-cultured myocardial cells was observed (Fig. 8A).

0073

MOMCs labeled with PKH67 (Green) expressed myocardial cell-specific transcription factors Nkx2.5, eHAND (Red/Alexa568: Molecular Probe), and expressed simultaneously CD45, a hematopoietic marker (Blue/Alexa660: Molecular Probe) (Figs. 8B, C). This shows the differentiating process of MOMCs derived from human peripheral blood hemocyte into myocardial cells.

0074

By RT-PCR using human-specific PCR primer, expression of myocin light chain (MLC2v) which is a myocardial cell structural protein was observed in human cardiac muscle, the positive control,

while no expression was observed in rat-cardiac muscle, then negative control, nor in MOMCs before coculture. In MOMCs at Day 12 of coculture, expression of human MLC2v was observed (Fig. 8D).

0075

From these results, with the coculture of MOMCs with rat-myocardial cells, induction of differentiation of MOMCs into myocardial cells was demonstrated, as well as the expression of myocardial progenitor marker at Day 8-10 of coculture, and the expression of myocardial structural protein at Day 12-14.

0076

Example 24 (In vitro differentiation of MOMCs into neurons)

Nestin (Brown), a marker expressing in nerve and myocardial progenitors, was expressed in MOMCs at Day 8 of coculture, and elongation of nerve projection toward surrounding rat-cultured neurons was observed (Fig. 9A).

0077

MOMCs labeled with PKH67 (Green) expressed a neuron-specific transcription factor NeuroD (Red/Alexa568: Molecular Probe) at Day 4 of coculture, and expressed simultaneously CD45 a hematopoietic marker (Blue/Alexa660: Molecular Probe) (Fig. 9B). This shows the differentiating process of MOMCs derived from human peripheral blood hematopoietic cells into neurons.

0078

At Day 3 of coculture, at the same time MOMCs expressed nestin (Green/Alexa488: Molecular Probe), Neurogenin2 (red/Alexa568: Molecular Probe), which is a neuron-specific transcription factor was expressed (Fig. 9C). From these results, MOMCs were shown to be a progenitor of neurons, whose differentiation into neuron is determined.

0079

With 9 days of coculture with Wistar rat-cultured neuron, expression of mature nerve markers Hu, NeuN (Red/Alexa568: Molecular Probe) was observed (Figs. 9D, and E) in MOMCs labeled with PKH67 (Green). These results demonstrated that MOMCs differentiate up to mature neurons.

0080

Example 25 (In vitro differentiation of MOMCs into endothelial cells)

MOMCs that underwent induction of differentiation in EBM-2 medium for 7 days, changed their morphology from spindle shape to a morphology having multiple projections, and expressed vWF, eNOS, VEGFR2/KDR/Flk-1 specific to endothelial cells that were not expressed originally.

0081

From a gene expression analysis by RT-PCR, in MOMCs that underwent induction of differentiation in EMB-2 medium for 7 days, expression of genes Flt-1, VEGFR2/KDR/Flk-1, CD31, CD144, vWf characteristic to endothelium was observed. Expression of Flt-1 and CD31 was observed in MOMCs, but other genes were expressed after the induction. After inducing differentiation, CD34 expression was enhanced, and expression of CD45, CD 14 was lost.

0082

From these results, it was demonstrated that by culturing MOMCs in maintenance medium of endothelial cells, differentiation into endothelial cells was induced.

Effect of the Invention

0083

According to the present invention, a monocyte-derived multipotent cell, MOMC, having a potential to differentiate into various cell types such as mesenchymal cells including bone, cartilage,

skeletal muscle and fat, endothelial cells, myocardial cells, neurons, very useful to cell therapy or regenerative medicine can be obtained. Moreover, as monocytes can be easily obtained without much invasion from peripheral blood, and monocytes represent about 20% of the peripheral blood mononuclear cells, sufficient cells necessary can be supplied in a stable manner. Inducing differentiation from monocytes into MOMCs can be performed easily, rapidly at a low cost, and no particular device is needed. Further, as autologous cells can be used for cell transplantation, there are no problems such as securing donors, or rejection symptoms, and fewer ethical problems. The present invention challenges the traditional and biological view regarding the monocyte/phagocyte systems and will contribute greatly to the understanding of the differentiation potential of monocytes and the roles they play for retaining homeostasis of the living body and induction of pathology.

0084

Sequence listing

<110> KEIO UNIVERSITY

<120> monocyte-derived multipotent cell MOMC

<130> 000000448

<160> 34

<170> PatentIn version 3.1

<210> 1

<211> 20

<212> DNA

<213> Artificial

<220>

<223> MLC2v-sense primer

<400> 1

tgacaagaac gatctgagag

20

<210> 2

<211> 20

<212> DNA

<213> Artificial

<220>

<223> MLC2v-antisense primer

<400> 2

caggttcttg tagtccaagt

20

<210> 3

<211> 21

<212> DNA

<213> Artificial

<220>

<223> Osterix-sense primer

<400> 3	
cttgtgcctg atacctgcac t	21
<210> 4	
<211> 22	
<212> DNA	
<213> Artificial	
<220>	
<223> Osterix-antisense primer	
<400> 4	
tcactctacc tgacccgta tc	22
<210> 5	
<211> 20	
<212> DNA	
<213> Artificial	
<220>	
<223> Bone sialoprotein II-sense primer	
<400> 5	
aaacggcacc agtaccaaca	20
<210> 6	

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Bone sialoprotein II-antisense primer

<400> 6

gccatcgtag cctgtcctt

20

<210> 7

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Osteocalcin-sense primer

<400> 7

gccatcgtag cctgtcctt

20

<210> 8

<211> 22

<212> DNA

<213> Artificial

<220>

<223> Osteocalcin-antisense primer

<400> 8
ggcagcgagg tagtgaagag ac 22

<210> 9
<211> 20
<212> DNA
<213> Artificial

<220>
<223> SkM-MHC-sense primer

<400> 9
ataggaacac ccaagccatc 20

<210> 10
<211> 20
<212> DNA
<213> Artificial

<220>
<223> SkM-MHC-antisense primer

<400> 10
ataggaacac ccaagccatc 20

<210> 11

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Myogenin-sense primer

<400> 11

tggccttccc agatgaaacc

20

<210> 12

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Myogenin-antisense primer

<400> 12

gcatcgggaa gagaccagaa

20

<210> 13

<211> 20

<212> DNA

<213> Artificial

<220>

<223> alpha1(II) collagen-sense primer

<400> 13

gcatcgggaa gagaccagaa

20

<210> 14

<211> 20

<212> DNA

<213> Artificial

<220>

<223> alpha1(II) collagen-antisense primer

<400> 14

agagtcctag agtgactgag

20

<210> 15

<211> 23

<212> DNA

<213> Artificial

<220>

<223> alpha1(X) collagen-sense primer

<400> 15

aatccctgga ccggctggaa ttc

23

<210> 16

<211> 23

<212> DNA

<213> Artificial

<220>

<223> alpha1(X) collagen-antisense primer

<400> 16

ttgatgcctg gctgtcctgg acc

23

<210> 17

<211> 20

<212> DNA

<213> Artificial

<220>

<223> PPARgamma-sense primer

<400> 17

aggagcagag caaagagtg

20

<210> 18

<211> 20

<212> DNA

<213> Artificial

<220>

<223> PPARgamma-antisense primer

<400> 18
aggactcagg gtggttcagg 20

<210> 19
<211> 22
<212> DNA
<213> Artificial

<220>
<223> aP2-sense-primer

<400> 19
tatgaaagaa gtaggagtgg gc 22

<210> 20
<211> 22
<212> DNA
<213> Artificial

<220>
<223> aP2-antisense-primer

<400> 20
ccaccaccag ttatcatcc tc 22

<210> 21

<211> 20

<212> DNA

<213> Artificial

<220>

<223> CD34-sense primer

<400> 21

cctcccaagt ttaggacaa

20

<210> 22

<211> 20

<212> DNA

<213> Artificial

<220>

<223> CD34-antisense primer

<400> 22

cagctggtag taagggttag

20

<210> 23

<211> 21

<212> DNA

<213> Artificial

<220>

<223> CD45-sense primer

<400> 23

aacctgaagt gatgattgct g

21

<210> 24

<211> 20

<212> DNA

<213> Artificial

<220>

<223> CD45-antisense primer

<400> 24

tacctcttct gtttccgcac

20

<210> 25

<211> 20

<212> DNA

<213> Artificial

<220>

<223> CD14-sense primer

<400> 25

ctgcgtgtgc tagcgtactc

20

<210> 26

<211> 20

<212> DNA

<213> Artificial

<220>

<223> CD14-antisense primer

<400> 26

cgtccagtgt caggttatcc

20

<210> 27

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Cbfa1/Runx2-sense primer

<400> 27

gtcttaccce tctacctga

20

<210> 28

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Cbfa1/Runx2-antisense primer

<400> 28

tgcctggctc ttcttactga

20

<210> 29

<211> 22

<212> DNA

<213> Artificial

<220>

<223> MyoD-sense primer

<400> 29

cctagactac ctgtccagca tc

22

<210> 30

<211> 20

<212> DNA

<213> Artificial

<220>

<223> MyoD-antisense primer

<400> 30

ggcggaaact tcagttctcc

20

<210> 31

<211> 21

<212> DNA

<213> Artificial

<220>

<223> Sox-9-sense primer

<400> 31

cccgatctga agaaggagag c

21

<210> 32

<211> 21

<212> DNA

<213> Artificial

<220>

<223> Sox-9-antisense primer

<400> 32

gttcttcacc gacttcctcc g

21

<210> 33

<211> 19

<212> DNA

<213> Artificial

<220>

<223> GAPDH-sense primer

<400> 33

tgaacgggaa gctcactgg

19

<210> 34

<211> 20

<212> DNA

<213> Artificial

<220>

<223> GAPDH-antisense primer

<400> 34

tccaccaccc tgttgctgta

20

Brief Explanation of Drawings

Fig. 1

Fig. 1 is a picture showing the morphology of MOMCs of the present invention. (a, b) PBMCs were cultured on fibronectin in low-glucose DMEM supplemented with 10% FBS for 7 days, and observed with a phase contrast microscope. The original magnifications are $\times 80$ for a, and $\times 40$ for b. MOMCs were moved on a new fibronectin-coated plate and cultured for 24 hours. (c) A picture showing the observation result with a phase contrast microscope ($\times 40$). (d, e) Pictures showing observation results with an electron microscope (d and e, $\times 5000$; f and g, $\times 30000$). A bundle of intermediate filaments is shown by an arrow, and a structure similar to a rod-shaped microtubulated body is shown by an arrowhead. L is for labyrinth-like endocytic vesicle;

LD is for lipid droplet; N is for nucleus, and PS is for pseudopodia.

Fig. 2

Fig. 2 is a figure showing the result of flow cytometric analysis of MOMCs of the present invention. PMBCs were cultured on fibronectin-coated plates and the adherent cells were harvested on Day 7. The cells were stained with a series of mAbs shown in the picture and analyzed by flow cytometry. The expression of the molecule of interest is shown as shaded histograms. Open histograms represent controls stained with isotype-matched control mAbs. The results shown are representative of at least three experiments.

Fig. 3

Fig. 3 is a picture explaining that MOMCs of the present invention originate from circulating CD14⁺ monocytes. (a) PBMCs were cultured on fibronectin for 1 hour, and 1, 3, 5, 7, 10, and 14 days. The adherent cells were harvested and stained with FITC-conjugated anti-CD14 and PC5-conjugated anti-CD34 mAbs and analyzed by flow cytometry. The results shown are representative of three independent experiments. (b) PBMCs depleted of CD14⁺ cells, CD34⁺ cells or CD105⁺ cells, and mock-treated PBMCs were cultured on fibronectin for 7 days. The number of attaching cells per cm³ was counted, and the results are expressed as the ratio to the number of attached cells in the untreated PBMC culture. The results shown are the mean and SD from three donors. The asterisk in the figure indicates a significant difference compared with mock-treated PBMC cultures. (c) MACS (Magnetic Cell Sorting)-sorted CD14⁺ monocytes were stained with PKH67 and cultured with or without unlabeled CD14⁻ cells (ratio 1:4) on fibronectin-coated or uncoated plastic plates for 7 days. The adherent cells were harvested, stained with PC5-conjugated

anti-CD34 mAb, and analyzed by flow cytometry. The results shown are representative of three experiments. (d) MACS-sorted CD14⁺ monocytes were stained with CFSE and cultured on fibronectin for 0, 1, 3, and 5 days. The adherent cells were harvested and stained with PC5-conjugated anti-CD14 mAb. The cells were analyzed by flow cytometry. The results shown are representative of three experiments.

Fig. 4

Fig. 4 is a picture showing the result of immunohistochemical analysis of MOMCs of the present invention. MOMCs generated by culturing PBMCs on fibronectin-coated plates for 7 days were moved onto fibronectin-coated chamber slides (The slides coated with type I collagen were only used for fibronectin staining). After 24 hours of culture, the above-mentioned slides were fixed with 10% formalin and stained with mAbs as indicated in the picture. The nuclei were counterstained with hematoxylin. Original magnification is $\times 100$. The results shown are representative of at least three independent experiments.

Fig. 5

Fig. 5 is a figure showing how the MOMCs of the present invention proliferate. MOMCs generated by culturing PBMCs on fibronectin-coated plates for 7 days were moved on fibronectin-coated chamber slides. MOMCs were further cultured for 1, 3, 5, 7, and 10 days, and stained with BrdU. The nuclei were counterstained with hematoxylin. (a) A representative figure at Day 1 and Day 5. The arrow indicates nuclei positive for BrdU staining. (b) At least 200 cells were counted to see the BrdU staining experiment result and the number of BrdU⁺ cells was calculated for individual slides cultured for 1, 3, 5, 7, and 10 days. The results shown are the mean and SD of five independent

experiments. (c) MOMCs were labeled with CFSE and were cultured on new fibronectin-coated plates for 0, 1, 3, 5 days. The adherent cells were collected and analyzed by flow cytometry. The results shown are representative of three independent experiments.

Fig. 6

Fig. 6 is a picture showing osteogenic, myogenic, chondrogenic, and adipogenic differentiation of MOMCs of the present invention. MOMCs before and after three weeks of osteogenic induction were stained with Alizarin red (a; magnification $\times 100$) or with alkaline phosphatase (b; $\times 100$). The intracellular calcium deposition was measured in MOMCs and fibroblasts before and after osteogenic induction and expressed as microgram per microgram protein content (c). Expression of mRNAs for osterix, bone sialoprotein II (BSP II), osteocalcin, CD34, CD45, CD14 and GAPDH were examined in MOMCs before and after 3 weeks of osteogenic induction and in osteosarcoma cell line (d). MOMCs before and after 3 weeks of myogenic induction were stained, and SkM-actin staining (e; $\times 200$) or SkM-MHC staining (f; $\times 200$) were examined. Expression of mRNAs for myogenin, SkM-MHC, CD34, CD45, CD14, and GAPDH was examined in MOMCs before and after 3 weeks of myogenic induction and in myoblast, muscle tissue, and in rhabdomyosarcoma cell line (g). MOMCs before and after 3 weeks of chondrogenic induction were stained and type II collagen staining were examined (h; $\times 40$). Expression of mRNAs for $\alpha 1$ (type II) and $\alpha 1$ (type X) collagen, CD34, CD45, CD14, and GAPDH was examined in MOMCs before and after 3 weeks of chondrogenic induction and in a chondrosarcoma cell line (i). MOMCs before and after 3 weeks of adipogenic induction were stained with Oil-red-O (j; $\times 200$). Expression of the mRNAs for PPAR γ , aP2, CD34, Cd45, CD14 and GAPDH was examined in MOMCs before and after three weeks of adipogenic induction

and in fat tissue (k). The results shown are representative of at least five experiments.

Fig. 7

Fig. 7 is a picture showing the coexpression of CD45 (green) and tissue-specific transcription factors (red) in MOMCs of the present invention that underwent 1 week of mesenchymal differentiation. MOMCs before induction treatment (a-d) and MOMCs treated for osteogenic (e), myogenic (f), chondrogenic (g) or adipogenic (h) induction for 1 week were examined for the immunohistochemical localization of CD45 in combination with Cbfa1/Runx2 (a and e), MyoD (b and f), Sox-9 (c and g), or PPAR γ (d and h). The cells were observed with confocal laser fluorescence microscopy (original magnification $\times 200$). The results shown are representative of three experiments.

Fig. 8

Fig. 8 is a picture showing how MOMCs of the present invention differentiate into myocardium. Nestins (brown) expressed in MOMCs cocultured with Wistar rat-cultured myocardial cells for 8 days were immunostained (A; $\times 200$). After labeling cell membrane with fluorescent PKH67 (green), MOMCs were cocultured with Wistar rat-cultured myocardial cells for 7 days, and were double fluorescently immunostained with Nkx 2.5 (red), a myocardial cell-specific transcription factor, and CD45 (blue), a hematopoietic marker (B; $\times 200$). After labeling cell membrane with fluorescent PKH67 (green), MOMCs were cocultured with Wistar rat-cultured myocardial cells for 8 days, and were double fluorescently immunostained with eHAND (red), a myocardial cell-specific transcription factor, and CD45 (blue), a hematopoietic marker (C; $\times 200$). MOMCs were cocultured with Wistar rat-cultured myocardial cells for 3, 6, 9, 12 days and expression

of the mRNAs for myosin light chain (MLC2v), a myocardial cell-structural protein (D). The results shown are representative of three experiments.

Fig. 9

Fig. 9 is a picture of how MOMCs of the present invention differentiate into neurons. Nestins (brown), which are markers expressing in nerve/myocardial progenitors, and being expressed in MOMCs cocultured with Wistar rat-cultured neurons for 8 days were immunostained (A; $\times 200$). After labeling cell membrane with fluorescent PKH67 (green), MOMCs were cocultured with Wistar rat-cultured neurons for 4 days, and were double fluorescently immunostained with NeuroD (red), a neuron-specific transcription factor, and CD45 (blue), a hematopoietic marker (B; $\times 200$). MOMCs were cocultured with Wistar rat-cultured neurons for 3 days, and were double fluorescently immunostained with nestin (brown) and Neurogenin 2 (red), a neuron-specific transcription factor (C; $\times 200$). MOMCs were cocultured with Wistar rat-cultured neuron for 3 days, and were double fluorescently immunostained with PKH67 (green) and Hu (red), a mature-nerve marker (D; $\times 200$). MOMCs were cocultured with Wistar rat-cultured neuron for 9 days, and were fluorescently immunostained with PKH67 (green) and NeuN (red), a mature-nerve marker (E; $\times 200$). The results shown are representative of three experiments.

Fig. 10

Fig. 10 is a picture showing how MOMCs of the present invention differentiate into endothelial cells. MOMCs induced to differentiate in a EBM-2 medium, a maintenance medium of endothelial cells, for 7 days, changed to a morphology having multiple projections from a spindle shape (left; $\times 200$). CD34 and endothelial cell-specific vWF, eNOS, VEGFR2/KDR/Flk-1, expressed

in MOMCs induced to differentiate in an EBM-2 medium for 7 days, were immunostained (brown) (middle; $\times 200$). Expression of the mRNAs for Flt-1, VEGFR2/KDR/Flk-1, CD31, CD144, vWF, CD34, CD45, CD14, and GAPDH, expressed in endothelial cells after 7-day culture of MOMCs in an EBM-2 medium, was examined (right). The results shown are representative of three experiments.

Name of Document DRAWING

Fig. 1

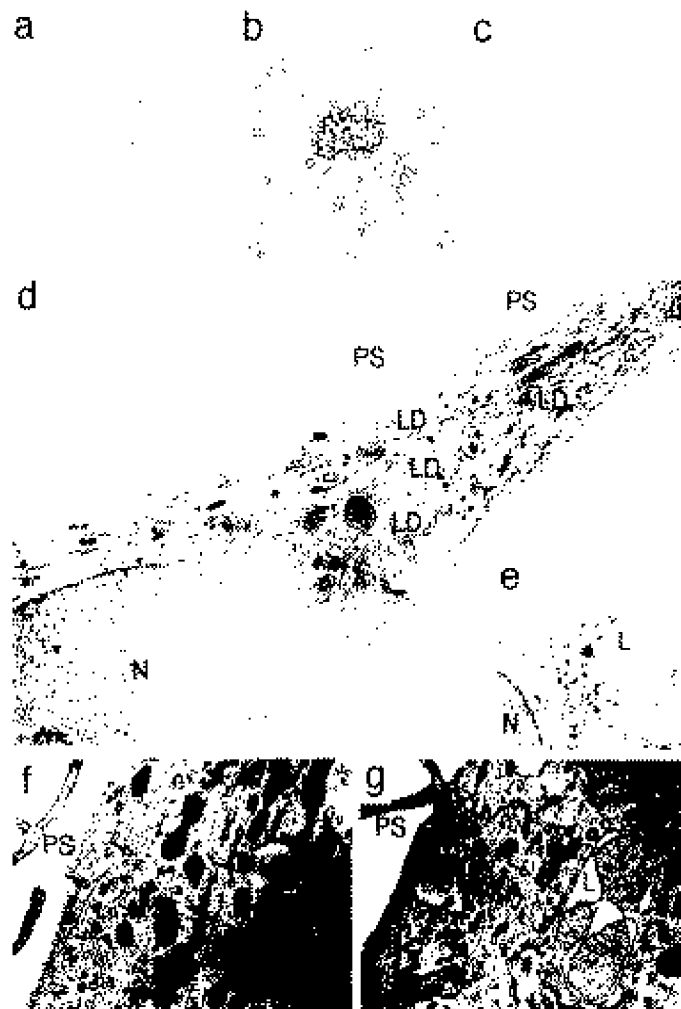


Fig. 2

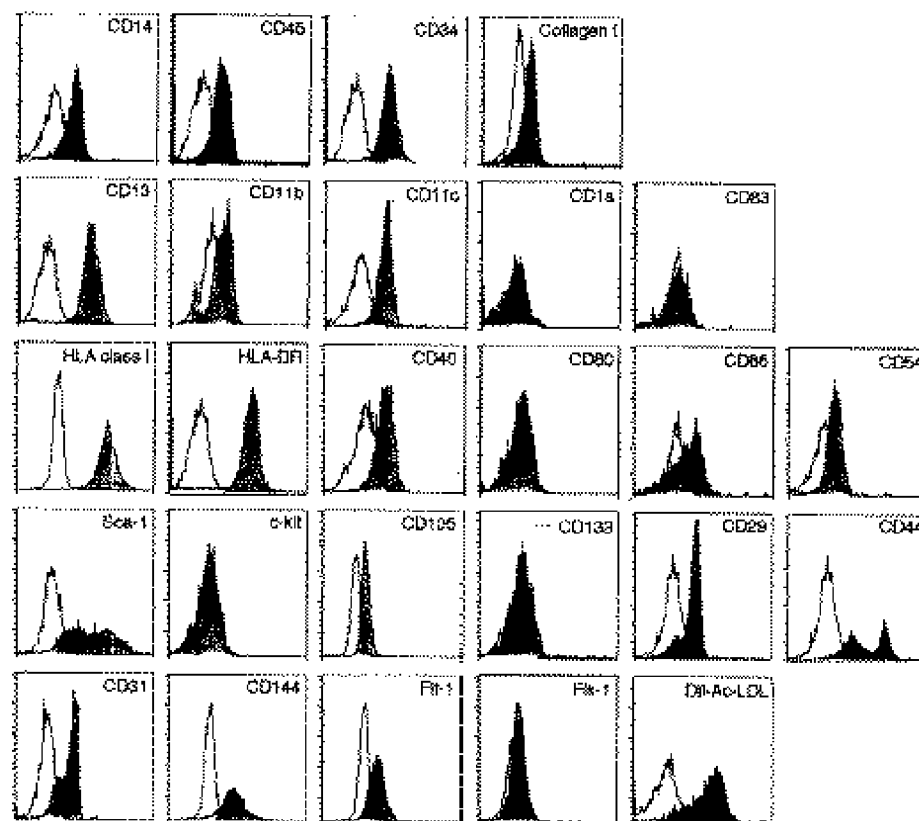


Fig. 3

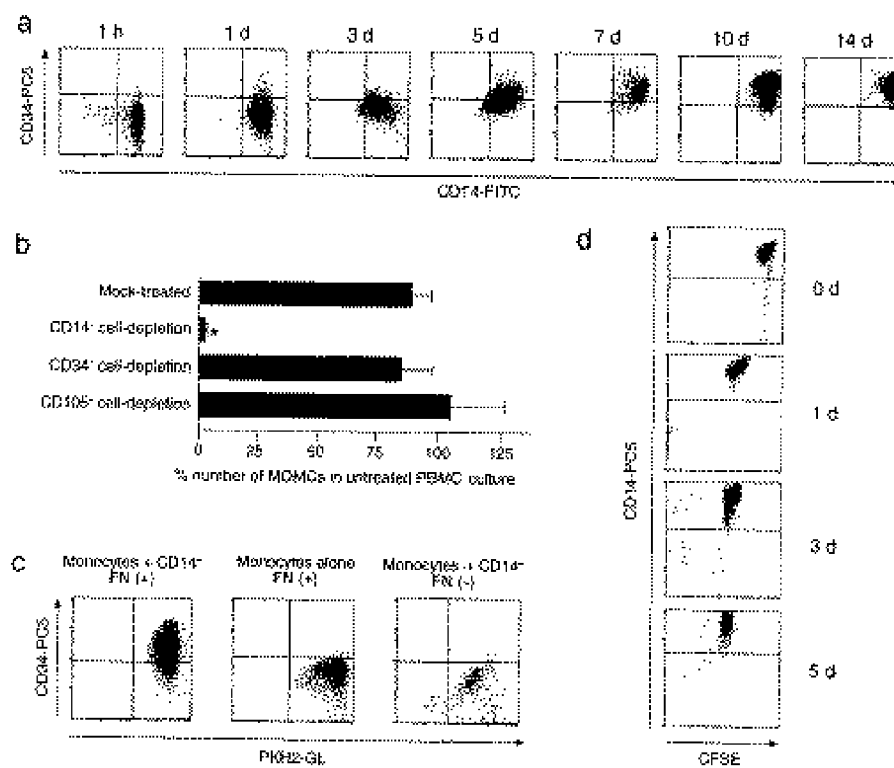


Fig. 4

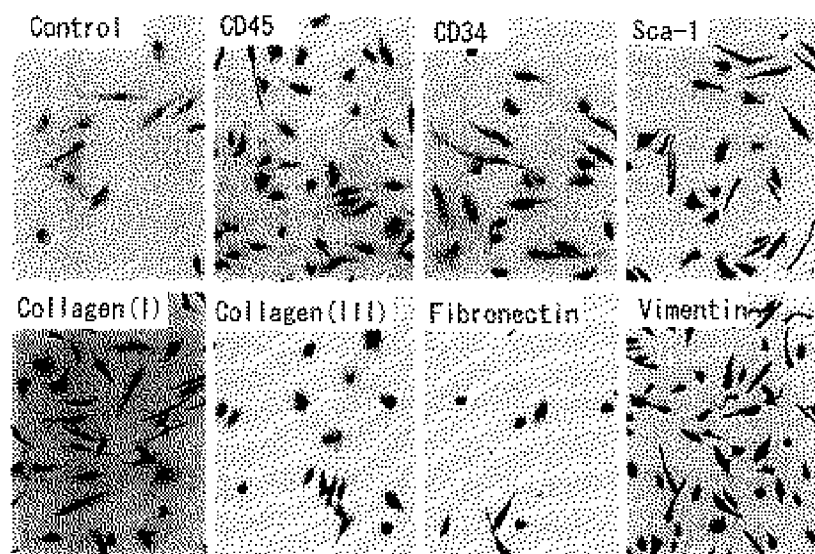


Fig. 5

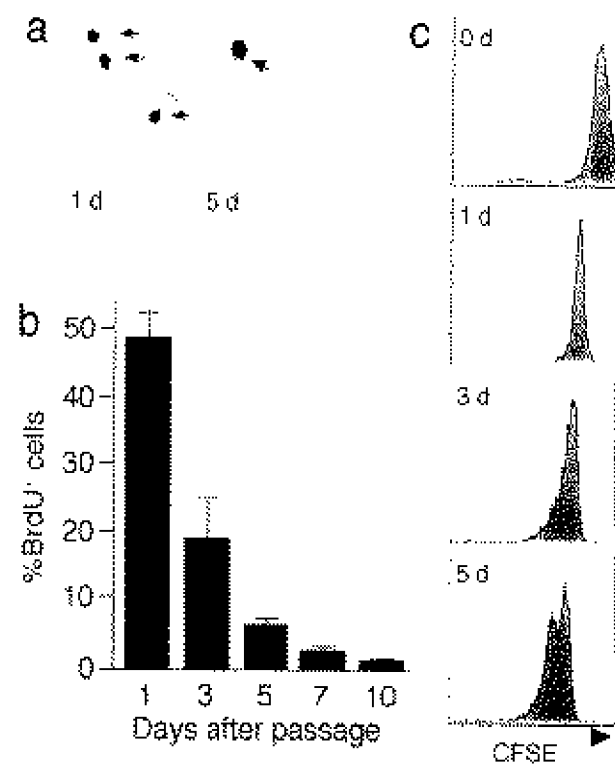


Fig. 6

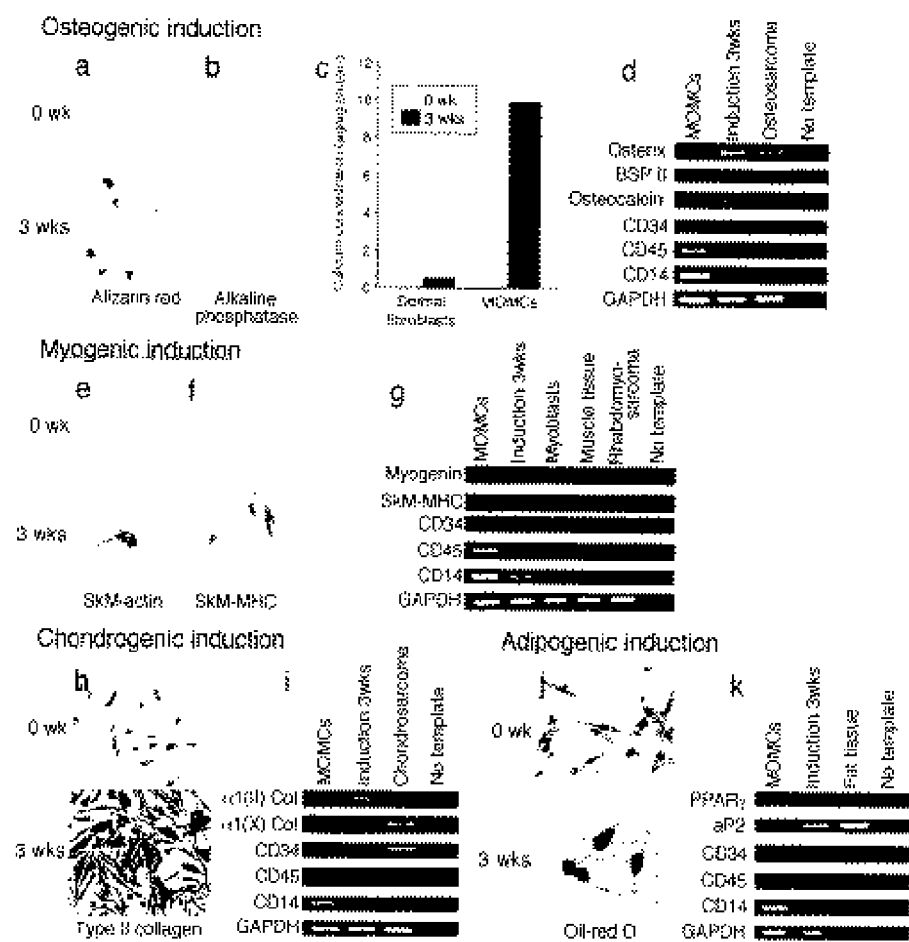


Fig. 7

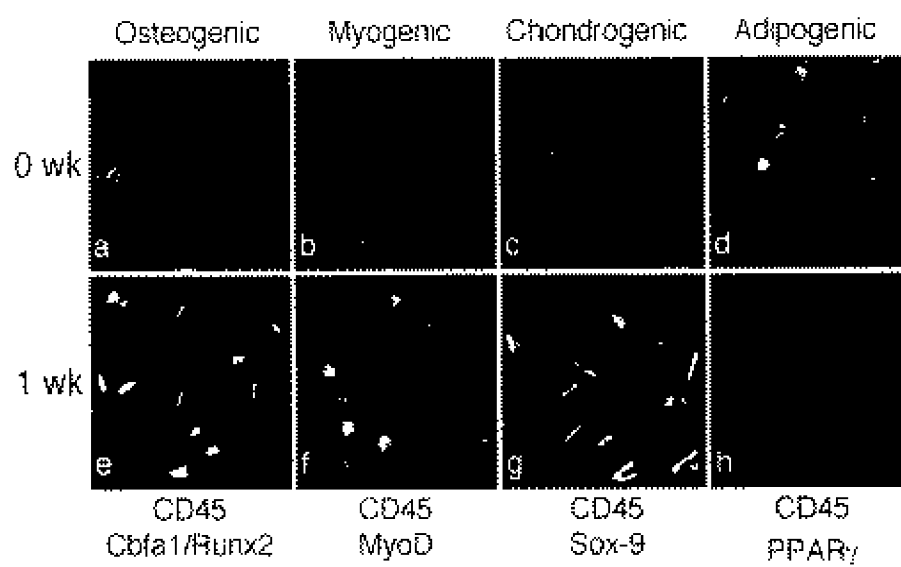


Fig. 8

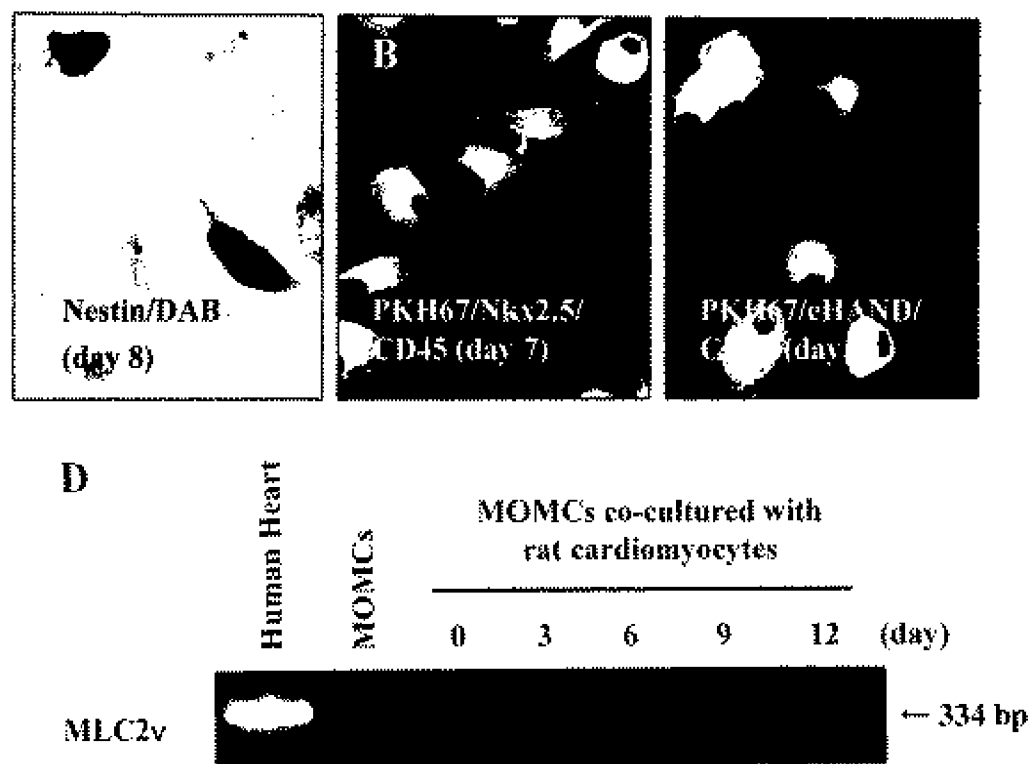


Fig. 9

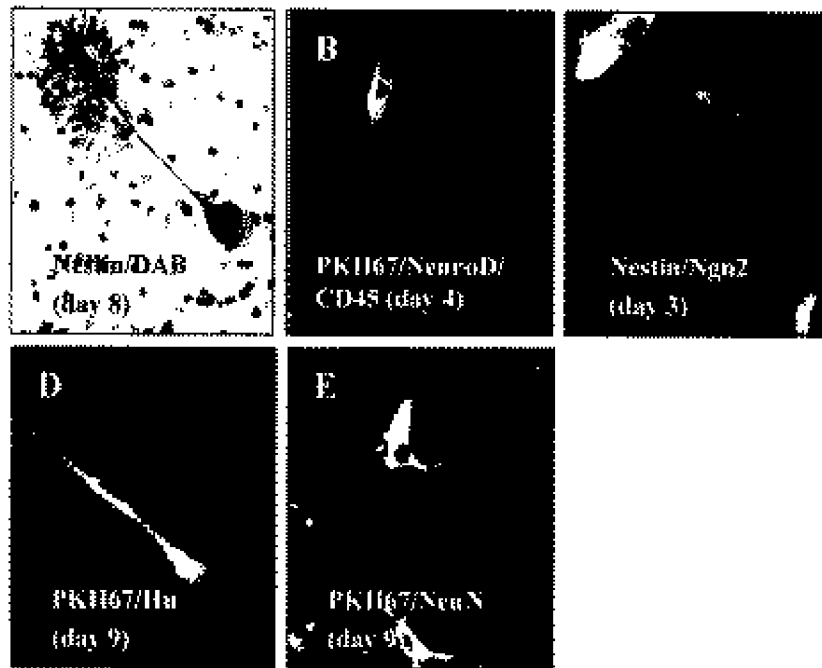
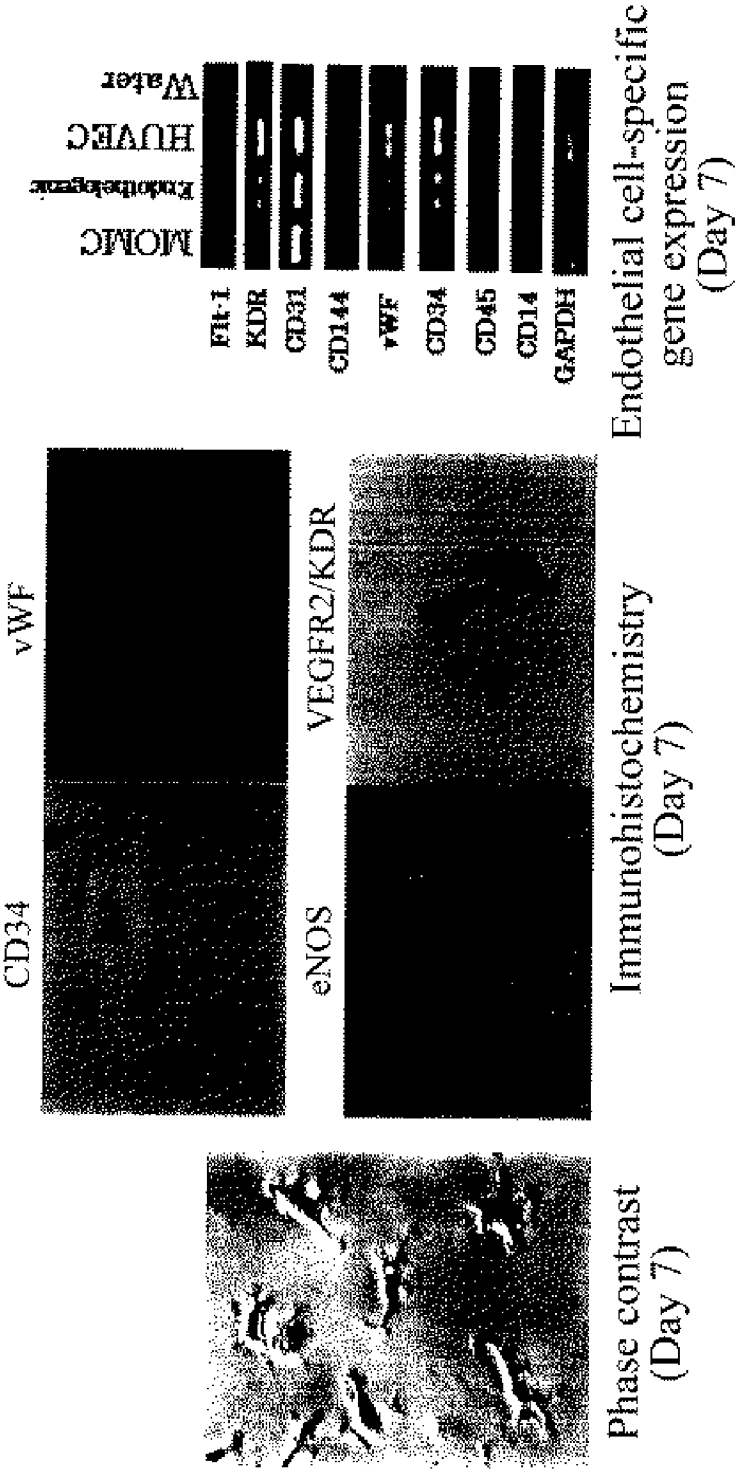


Fig. 10



Name of Document ABSTRACT

Abstract

The Object The object of the present invention is to provide multipotent cells wherein the sufficient amount necessary can be stably and conveniently supplied with a minimum invasion, that will not cause rejection at the time of cell transplantation, that have potentials to differentiate into various cells such as mesenchymal cells including bone, cartilage, skeletal muscle and fat, endothelial cells, myocardial cells, neurons; mesenchymal cells, myocardial cells, endothelial cells, neurons induced to differentiate from the multipotent cell, and a therapeutic agent/treating method comprising these as active ingredient.

Solving Means Peripheral blood mononuclear cells (PMBC) are cultured on fibronectin-coated plastic plates for 7 to 10 days. The generating cell population with a fibroblast-like morphology is derived from circulating CD14⁺ monocyte, with a unique phenotype of CD14⁺CD45⁺CD34⁺ type I collagen⁺. These cells have a potential to differentiate into mesenchymal cells including bone, cartilage, skeletal muscle and fat, endothelial cells, myocardial cells, and neurons under particular culture conditions.